Maternal effects of carotenoid consumption in guppies (*Poecilia reticulata*)

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Summary

1. Carotenoids transferred from mother to offspring may enhance the quality of the offspring. Whether such maternal effects occur in guppies (*Poecilia reticulata*) has an important bearing on mate preference evolution.
2. By raising female guppies from birth on different dietary carotenoid levels, we examined the pattern of carotenoid allocation to maternal tissue (skin) vs. eggs. Skin carotenoid content was only weakly affected by carotenoid intake while egg carotenoid content was strongly affected.
3. We then tested for effects of maternal carotenoid intake on several measures of offspring quality, including size and condition at birth, juvenile growth rate, and the size, condition, skin carotenoid content and colouration of mature sons. To test for interactions between maternal and offspring carotenoid intake, broods were split and offspring were reared on one of two carotenoid levels.
4. Offspring carotenoid intake had the expected effects on male colouration, but otherwise we found no evidence that maternal or offspring carotenoid intake influences offspring quality. It remains possible that maternal carotenoids affect offspring fitness parameters that we did not measure or that such effects depend on environmental factors that were absent in our laboratory aquaria.
5. Our review of the literature on maternal carotenoid effects in birds and fishes suggests that such effects may be taxon-specific. Thus, it seems unwarranted to assume that an adaptive trade-off necessarily exists between allocation of carotenoids to eggs vs. maternal tissues. Alternative hypotheses, such as the possibility that eggs provide a means of excreting excess carotenoids, also merit consideration.
6. Our results indirectly support the indicator model of mate preference evolution by casting doubt on an alternative hypothesis that requires females to benefit more from consuming carotenoids than males do.

Key-words: egg carotenoids, maternal effects, mate preference, offspring quality

Introduction

Maternal-environmental effects, or effects of a mother's environment on her offspring's phenotype, can have important ecological and evolutionary consequences (Qvarnstrom & Price 2001; Benton *et al*. 2005). Maternal contributions to offspring can include carotenoids and other molecules with antioxidant properties that may help protect developing embryos from oxidative stress (reviewed in Blount, Houston & Moller 2000; McGraw, Adkins-Regan & Parker 2005; Bertrand *et al*. 2006; Groothuis *et al*. 2006; but see Hartley & Kennedy 2004). In species with carotenoid-based sexual colouration, maternal carotenoids may also influence the colouration and hence mating success of offspring (especially sons; McGraw *et al*. 2005). Animals cannot synthesize carotenoids (Goodwin 1984), and thus, in environments in which carotenoids are a limiting resource, a female's ability to consume carotenoid-rich foods may influence the quality of her offspring. Moreover, a trade-off may exist between the allocation of carotenoids to maternal vs. offspring tissues. The best evidence for maternal carotenoid effects comes from studies on birds in which the carotenoid content of egg yolks was manipulated experimentally, either by varying the carotenoid content of the maternal diet or by injecting carotenoids directly into the yolks (e.g. Saino *et al*. 2003; McGraw *et al*. 2005).

We tested for maternal effects of carotenoid consumption in guppies (*Poecilia reticulata*), a species of freshwater fish that experiences varying degrees of carotenoid limitation in nature (Grether, Hudon & Millie 1999). Whether carotenoid consumption is beneficial for female guppies, either directly or through effects on offspring fitness, has an important

bearing on mate preference evolution in this species. Guppies of both sexes are innately attracted to small orange objects and this sensory bias appears to be genetically linked to the female preference for orange colouration in males (Rodd *et al*. 2002; Grether *et al*. 2005b). In addition to covarying geographically, orange attraction and the associated female preference were both enhanced by raising females on a low-carotenoid diet (Grether *et al*. 2005b). This plastic response could have evolved either as a foraging adaptation or through sexual selection (i.e. the indicator process; Grether 2000). As a foraging adaptation, it might serve to cause females to increase consumption of carotenoid-rich orange fruits (e.g. *Slonea laurifolia*) when other dietary sources of carotenoids are scarce. A problem for this hypothesis is that carotenoid-dependent expression of the sensory bias was not found in males (Grether *et al*. 2005b). However, if maternal carotenoid consumption has strong, positive effects on female fecundity or offspring quality, then perhaps females benefit more from consuming carotenoids than males do.

Our primary goal in this study was to test for effects of carotenoid consumption on female fecundity and offspring quality. A secondary goal was to examine the potential trade-off between carotenoid allocation to maternal vs. offspring tissues. First, after showing that female guppies deposit carotenoids in their eggs, we examine how the level of carotenoids in the maternal diet affects allocation of carotenoids to maternal skin vs. eggs. Second, we test for effects of maternal and offspring carotenoid diet on female fecundity and a suite of offspring traits, including size and condition at birth, juvenile growth rate, and the size, condition, skin carotenoid content and colouration of mature males. To our knowledge, this is the most comprehensive study of maternal carotenoid effects yet undertaken in a fish.

Materials and methods

STUDY POPULATIONS

Fish used in this experiment were first or second generation laboratory-born descendents of wild adults collected from four streams in the Northern Range of Trinidad (Aqui River [Universal Transverse Mercator Grid coordinates, Zone 20: PS 939·2 886·6] and a smaller tributary [PS 950·1 880·0] of the Madamas River in the upper Madamas drainage; Small Crayfish River [PS 970·7 835·2] and Large Crayfish River [PS 696·5 832·2] in the upper Quare drainage). Fish were housed on the UCLA campus in a windowless room maintained on a 12 : 12 photoperiod (mixed incandescent and daylight spectrum fluorescent lights). Water was maintained at 24 ± 1·5 ºC and pH 7·4–7·9, simulating conditions in nature, and treated with 2-chloro-4,6-bis-(ethylamino)-s-triazine (Algae Destroyer, Aquarium Pharmaceuticals, Chalfont, PA) to retard algae growth; any visible algae were removed regularly. All protocols were approved by the UCLA Institutional Animal Care and Use Committee.

EXPERIMENT 1

This experiment was designed to test for effects of carotenoid consumption on carotenoid deposition in the eggs and skin of females. Guppies are lecithotrophic livebearers; embryos develop inside the female for about 25 days before parturition but receive no maternal provisioning beyond that deposited in the egg yolk (Reznick, Callahan & Llauredo 1996). Thus, any carotenoids passed from mother to offspring would be present in eggs. Two dietary carotenoid concentrations (trace and high) were crossed with two food levels (low and high) to test for interactions between food availability and carotenoid intake. Females were raised from birth on one of the four diets (see below for more information on the diets), sexed before sexual maturity, housed in group tanks separately from males, and then placed in a 180-L tank with adult males for 48 h after reaching maturity as part of a concurrent study on mate choice (see Grether *et al*. 2005b). Immediately after the mating trials, females were weighed to 0·01 mg, measured with calipers (standard length from lower jaw to caudal peduncle) to 0·01 mm, euthanized with anaesthetic (MS-222), frozen in liquid nitrogen, and stored at –80 ºC for skin and egg carotenoid extractions.

EXPERIMENT 2

Experiment 1 showed that the carotenoid concentration of the diet strongly influenced the carotenoid content of the eggs but that food availability had no significant effect after controlling for body mass (see Results). Experiment 2 was designed to test for effects of the same two dietary carotenoid levels on female fecundity and the following offspring traits: size and condition at birth, juvenile growth rate, and size, condition, skin carotenoid content and colouration of mature males. Females to be used as dams in this experiment were raised from birth on the high food level (to control for food availability) and one of the two dietary carotenoid concentrations, sexed, housed in group tanks separately from males until reaching sexual maturity, and then placed in an 8-L tank with a single adult male from the same population. Pairs were maintained on the female's diet treatment. Females usually give birth about 1 month after first being placed with a male. Because adult guppies sometimes cannibalize their own offspring, tanks were equipped with mesh dividers and *Fontinalis* moss to provide refuges (mesh restricted adults to one side of the tank but permitted young to move freely). Tanks were checked at least once per day and offspring were immediately separated from parents and moved to empty 8-L tanks. We divided each litter as evenly as possible into two sub-litters, randomly assigning one sub-litter to the trace-carotenoid diet and the other to the high-carotenoid diet. We considered a litter to be usable if we obtained growth rate estimates (see below) on offspring from both sub-litters. If a female's first litter was unusable, her second litter was used instead. *Fontinalis* was not an uncontrolled source of carotenoids in this experiment; guppies do not appear to eat it and in any case it was not present in the offspring tanks or the tanks in which dams were housed prior to mating.

EXPERIMENTAL DIETS

The high food level (used in both experiments) was about as much ground flake food as guppies of a given age were willing to eat on a twice-daily feeding schedule (as determined in pilot feeding trials based on the presence of uneaten food). The low food level (used only in Experiment 1) was one-third of the high food level. At each feeding, the quantity of food delivered to a tank was directly proportional to the number of fish in the tank. Food amounts increased on a predetermined schedule as the fish aged (see Kolluru *et al*. 2006b for further details). Comparisons of mature male size in the field and laboratory indicate that the high food level was in the middle

of the range that guppies typically experience in the wild, while the low food level was on the low end of the range (Kolluru *et al.* 2006a). The carotenoid diets were designed to contain carotenoid pigments found in the natural diets of guppies (see Kolluru *et al.* 2006b for details). Judging from the skin carotenoid content of males raised on these diets, the trace-carotenoid diet was lower in carotenoids than guppies encounter in nature while high-carotenoid diet was at the upper end of the natural range (Kolluru *et al.* 2006a).

OFFSPRING GROWTH RATES

Offspring were lightly sedated with MS-222 for length and weight measurements at 3, 15 and 50 days after birth and every 2 weeks thereafter until the sexes could be easily distinguished. Siblings were not distinguished individually prior to sexing and thus measurements were averaged to yield a mean standard length and weight for each sub-litter at each measurement date. Examination of growth trajectories of individual sub-litters showed that growth was approximately linear between 3 and 70 days of age, both in terms of standard length (mm/day) and body mass (mg/day). We therefore estimated juvenile growth rate from the slope of the linear regression of standard length or body mass on measurement age ≤ 70 days (mean adjusted-$R^2 \pm$ SE: $0{\cdot}96 \pm 0{\cdot}01$ and $0{\cdot}95 \pm 0{\cdot}01$ for standard length and body mass, respectively).

COLOUR MEASUREMENTS

After male offspring developed mature gonopodia and colouration, they were lightly sedated with MS-222 and photographed on both sides of the body with a digital camera (Olympus C2500L, Center Valley, PA) under standardized lighting (two tungsten-halogen fibre optic illuminators pointing at the dorsal side of the fish from 45° relative to the lateral surface). Image analysis software (NIHIMAGE 1·62; http://rsb.info.nih.gov/nih-image/) was used to measure the area of body, tail (caudal fin) and all colour pattern elements. The total area of each colour class on the body and tail was calculated and converted to proportions of body and tail area for analysis. Six colour classes were distinguished in the analysis: orange, yellow, green/blue, violet, white and black (melanin).

Immediately after photographs were taken, the reflectance spectrum (300–700 nm) of a specific set of colour spots on each fish was measured with a diode-array spectrometer (Ocean Optics S2000; Dunedin, FL) using a reflectance probe (Ocean Optics R-400) and xenon light source (Ocean Optics PX-2). Individual colour spots were isolated by holding them up to a 1·3-mm diameter aperture in a horizontally mounted, razor-thin, black anodized steel plate with the reflectance probe oriented 45° relative to the upper surface of the plate at the distance where the aperture edges matched the acceptance angle of the detector fibre. Fish were held with their long axis perpendicular to, and their dorsum facing, the light path. Reflectance was calculated relative to a white standard (Ocean Optics WS-1). The set of colour spots scanned on each fish included all orange spots on the body (excluding fins) and one green/blue and one ultraviolet-violet spot on each side of the body (if present). Each spot was scanned three times and replicate spectra were averaged.

Reflectance spectra were converted into guppy-specific cone excitation estimates (E_j) using the known λ_{max} values of guppy cones, as described in Grether, Cummings & Hudon (2005a). E_j were used to calculate relative cone contrasts and coordinates in tetrahedral colour space, following Endler & Meilke (2005). The sum of E_j across the four photoreceptor cone classes provides an estimate of luminance (perceived brightness). E_j calculations require that the ambient light (irradiance) spectrum be specified. The results presented below were qualitatively the same (at $\alpha = 0{\cdot}05$) for the five forest light environments characterized by Endler (1993). For brevity, we present results for just one irradiance spectrum (open-cloudy).

After the colour measurements, fish were weighed, measured with calipers, euthanized with MS-222 and frozen instantly in liquid nitrogen for pigment analysis.

PIGMENT ANALYSIS

The orange spots of guppies contain two classes of pigments: carotenoids and drosopterins (Grether, Hudon & Endler 2001). Skin pigment analyses were carried out as described in Grether *et al.* (2001) except that a different spectrophometry system was used. Absorbance spectra of hexane (carotenoid) or acidified ethanol (drosopterin) extracts were measured over 300–700 nm using a diode array spectrometer (Ocean Optics USB2000) with a cuvette holder attached to a deuterium-tungsten light source (DT-1000). Egg masses were removed by dissection, mashed and extracted for 15 min in acetone. Acetone extracts were filtered (0·45 μm Millex HN nylon filter, Millipore Corp., Billerica, MA), evaporated under a stream of nitrogen, and redissolved in hexane. Carotenoid content of the hexane extracts was measured from the peak of absorbance between 437 and 449 nm using an extinction coefficient, $E^{1\%}$/1 cm, of 2350 (Britton 1985). Orange spot pigment concentrations were calculated by dividing pigment content by the area of the body covered with orange spots.

STATISTICAL METHODS

In Experiment 1, carotenoid diet (trace vs. high) was crossed with two food levels (low vs. high). In Experiment 2, maternal carotenoid diet was crossed with offspring carotenoid diet. A nearly balanced design was achieved with respect to population of origin and diet group in Experiment 1 but not in Experiment 2. We restricted most analyses of Experiment 2 data to the population for which we had the largest sample size (Small Crayfish River), to avoid confounding population differences with diet effects; for some analyses this restriction was unnecessary and population of origin was included as a fixed effect in the analysis.

In Experiment 1, we used two different methods to examine effects of the experimental diets on carotenoid deposition while controlling for female body size: (i) ANCOVA with log carotenoid content (μg) of skin or eggs as the dependent variable and log body mass as the covariate; and (ii) ANOVA with log carotenoid concentration of skin (μg/mm^2) as the dependent variable. Polynomial regression showed that log carotenoid content scales linearly with log body mass (not shown). Estimates of skin carotenoid concentration were obtained by dividing skin carotenoid content by body area, as estimated from the equation, body area = $-29{\cdot}06 + 5{\cdot}81$ (standard length) + $0{\cdot}42$ (body mass). This equation was obtained by regressing body mass and length on body surface area for a sample of guppies whose body area was estimated from digital photographs.

In Experiment 2, while testing for effects of the diet treatments on offspring size, condition and growth rate, we evaluated the usefulness of the following covariates: litter number (i.e. first or second), litter size, dam standard length, sire standard length, dam condition (body mass/standard length^3) and sire condition. To select covariates for a given dependent variable, we used a stepwise backward selection procedure with $P = 0{\cdot}1$ as the criterion for retention in the final

model. A standard assumption in ANCOVA is that covariates and main effects do not interact. We examined all two-way interactions between the selected covariates and diet treatment and none were significant at $\alpha = 0{\cdot}05$. We also tested for diet treatment effects without using covariates and obtained qualitatively the same results at $\alpha = 0{\cdot}05$ (not shown). To take into account the split litter design, effects of maternal and offspring diet on growth rate were evaluated using MANCOVA with growth rate on the two offspring carotenoid levels as the dependent variables.

We could not use a nested design in the analysis of mature male traits because of a lack of replication within many sub-litters. We ran this analysis using individual males as sampling units and also using sub-litters as sampling units (i.e. averaging the data for males within sub-litters). The results of the two analyses were essentially the same; here we report results from the analysis with males as sampling units, noting where the sub-litter-based analysis gave a slightly different result. All continuous variables were log- or square-root transformed as needed to meet the assumptions of ANCOVA; residuals were tested for deviations from normality and homoscedasticity.

Effects of maternal and offspring diets on offspring colour would be relevant if and only if these effects were perceptible to guppies. In theory, all of the information available to tetrachromatic animals for making colour comparisons is captured by tetrahedral colour coordinates (Endler & Meilke 2005). Euclidian distances between points in this three-dimensional colour space provide an estimate of perceived colour contrast. To simultaneously test for the main effects of two factors and their interaction, one colour pattern element at a time, we used the Analysis of Distance (AOD) method, which is conceptually similar to MANOVA and compares favourably to MANOVA in power simulations, but does not rely on distributional assumptions (Gower & Krzanowski 1999; Fenty 2004). We ran AOD in STATA 9·2 (Stata Corporation, College Station, TX) with 10^4 permutations per run. Where significant treatment effects were found using AOD, we examined the nature of these effects at the level of relative cone contrasts.

Results

MATERNAL SKIN AND EGG CAROTENOIDS (EXPERIMENT 1)

Egg mass extracts showed the trimodal absorbance spectra characteristic of carotenoids (Fig. 1). The wavelength of peak absorbance of carotenoids in eggs was shifted upwards relative to that of carotenoids in the skin of the same females by 5·4 nm, on average (mean ± SE; egg: 446·8 ± 0·2; skin: 441·3 ± 0·1; paired t-test: $t = 25{\cdot}17$, df = 77, $P < 0{\cdot}0001$). This indicates that carotenoid composition differs between eggs and skin (see Discussion).

Females raised on the high-food level deposited significantly more carotenoids in their skin and eggs than low-food females (Fig. 2), but these differences disappeared after controlling for body mass (Table 1). Food level had no significant effect on the concentration of carotenoids in the skin (ng carotenoids per mm^2).

The concentration of carotenoids in the food affected skin carotenoid deposition only within the high-food level treatment, as reflected by a significant interaction between food level and carotenoid diet (Fig. 2; Table 1). Within the high-food group, females raised on the high-carotenoid diet had greater amounts (planned comparison, $t = 2{\cdot}44$, $P = 0{\cdot}017$) and concentrations ($t = 2{\cdot}48$, $P = 0{\cdot}015$) of carotenoids in their skin than females raised on the trace-carotenoid diet. A non-significant trend in the opposite direction was observed in the low-food group ($P > 0{\cdot}2$).

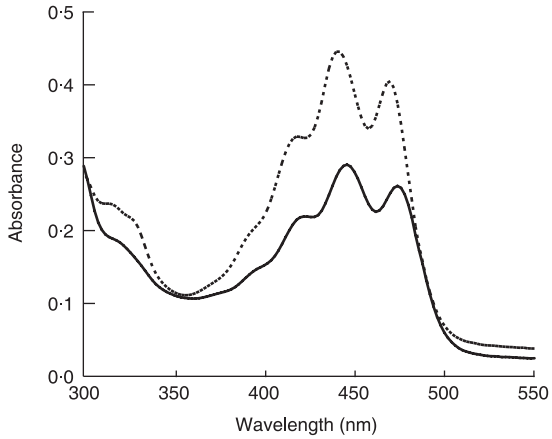

Fig. 1. Mean absorbance spectra of the carotenoids in the eggs (solid line) and skin (dotted line) of female guppies from a single population (Small Crayfish River). Based on $n = 26$ skin samples and $n = 20$ egg samples (Experiment 1).

Carotenoid deposition in the eggs was strongly affected by the carotenoid concentration of the food at both food levels (Fig. 2; Table 1). Females on the high-carotenoid diet deposited significantly larger amount of carotenoids into their eggs than did females on the trace-carotenoid diet, and this result was strengthened by including log body mass as a covariate. Including population of origin in the ANOVAs increased the R^2 values slightly (range of R^2-change values: 0·005–0·038), but did not alter the results with respect to diet effects (not shown). The population term was non-significant except in the skin carotenoid ANOVA ($P = 0{\cdot}047$).

These patterns of carotenoid deposition suggest that the carotenoid concentration of the food affects the proportional allocation of carotenoids to skin vs. eggs. This inference was borne out by an analysis of the egg : skin carotenoid ratio of individual females. Females in the high-carotenoid diet group allocated relatively more carotenoids to their eggs than did females in the trace-carotenoid diet group (Fig. 2; Table 1). There was no significant effect of food level on the egg : skin carotenoid ratio and no significant interaction between food level and carotenoid concentration.

Overall, across all diet groups, skin carotenoid content exceeded egg carotenoid content (paired t-test on log-transformed data, $t = 6{\cdot}05$, df = 77, $P < 0{\cdot}0001$), but this was not the case for all individual fish, and the difference was only significant within the trace-carotenoid diet groups (low-food-trace-carotenoid, $t = 3{\cdot}87$, df = 17, $P = 0{\cdot}0012$; high-food-trace-carotenoid, $t = 6{\cdot}90$, $n = 19$, $P < 0{\cdot}0001$; low-food-high-carotenoid, $t = 1{\cdot}60$, df = 20, $P = 0{\cdot}13$; high-food-high-carotenoid, $t = 1{\cdot}44$, df = 18, $P = 0{\cdot}17$).

In summary, carotenoid deposition in the skin was relatively insensitive to diet while egg carotenoid content was strongly

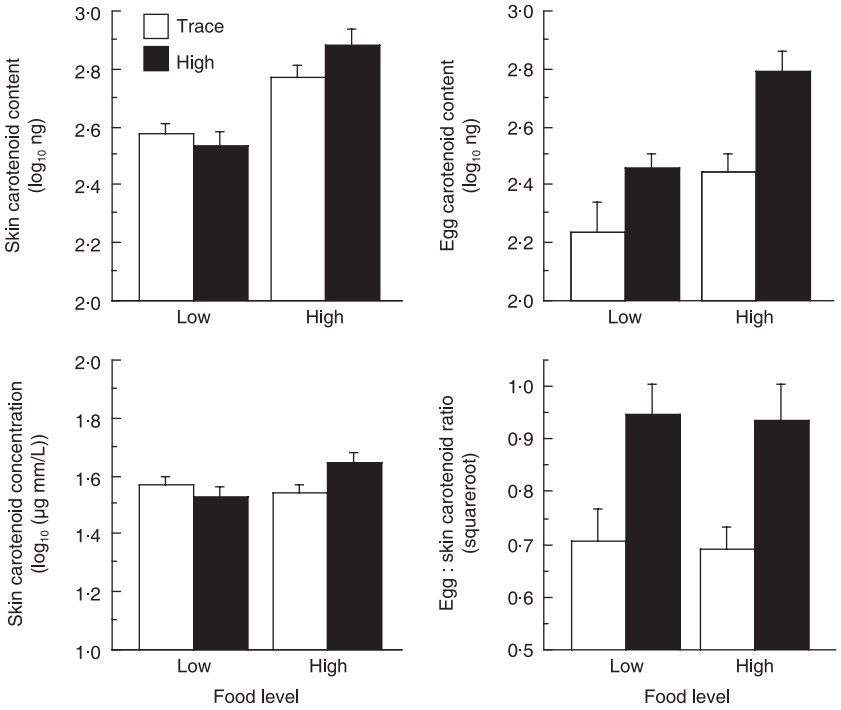

Fig. 2. Effects of dietary carotenoid concentration and food level on the levels and ratios of carotenoids in the eggs and skin of female guppies (Experiment 1). Results for females raised on the trace- and high-carotenoid diets are shown with unfilled (white) and solid (black) bars, respectively (means ± 1 SE). ANOVA results are shown in Table 1.

Table 1. Effects of experimental diets on carotenoid deposition in the skin and eggs of female guppies (Experiment 1). The direction of the effects are described in the text and the means are displayed in Fig. 2. Values shown are *F* statistics. All continuous variables were transformed to meet parametric assumptions of linearity, normality and homoscedascity of residuals. Egg : skin carotenoid ratio was square root-transformed; all other variables were log-transformed. Sample sizes are lower for eggs than skin because not all females contained eggs

Dependent variable (units)	Food level	Carotenoid concentration	Food × carotenoid	Body mass	R^2-adjusted	df
Skin carotenoid content (µg)	38·90****	0·76 ns	2·85 ns	–	0·29	1, 92
Skin carotenoid content (µg)	0·40 ns	0·88 ns	6·45*	112·41****	0·68	1, 91
Skin carotenoid concentration (ng/mm^2)	1·84 ns	1·16 ns	6·06*	–	0·06	1, 92
Egg carotenoid content (µg)	13·01***	14·75***	0·70 ns	–	0·25	1, 74
Egg carotenoid content (µg)	0·37 ns	20·55****	0·52 ns	30·66****	0·47	1, 73
Egg : skin carotenoid ratio	0·03 ns	16·67****	0·0006 ns	–	0·15	1, 74

ns, non-significant: $P > 0·1$, *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, ****$P < 0·0001$.

affected by the carotenoid concentration in the food. Most females allocated more carotenoids to their skin than to their eggs, but the proportional allocation of carotenoids to the eggs increased as the concentration of carotenoids in the diet increased.

MATERNAL FECUNDITY (EXPERIMENT 2)

About half of the females that we isolated with males gave birth during the experiment, which is similar to the rate reported by other researchers (e.g. Evans & Magurran 2000). Of the 41 females that did give birth, 37 (90·0%) also gave birth to a second litter. There were no significant effects of maternal carotenoid diet on the proportion of females that gave birth to first or second litters (Fishers exact tests, both $P > 0·9$). Litter size data were available for 18 additional first litters from a pilot phase of the experiment in which second litters were not collected. First litter size ranged from 1 to 12 offspring (mean ± SE: 3·83 ± 0·34; $n = 59$) and was not affected by the level of carotenoids in a female's diet or her population of origin and there was no significant interaction between these two factors (two-way ANOVA restricted to the two populations with replication in both diet groups [Madamas Tributary and Small Crayfish River]; carotenoid diet $F_{1,47} = 0·66$, $P = 0·42$; population $F_{1,47} = 0·05$, $P = 0·83$; interaction $F_{1,47} = 0·54$, $P = 0·46$). Second litter size also ranged from 1 to 12 offspring (mean ± SE: 4·32 ± 0·52; $n = 37$) and was not affected by the level of carotenoids in a female's diet or her population of origin (carotenoid diet $F_{1,25} = 1·68$, $P = 0·21$; population $F_{1,25} = 3·11$, $P = 0·09$; interaction $F_{1,25} = 0·05$, $P = 0·81$). The lack of a population difference in litter size is not a general result; litter size differs genetically between other guppy populations (e.g. Reznick & Endler 1982).

Table 2. Effects of maternal and offspring carotenoid diet on the pigmentation and orange spot luminance of mature male offspring (Experiment 2). The direction of the effects is described in the text. Pigment contents and concentrations were $\log_{10}$-transformed to meet parametric assumptions. Values shown are *F* statistics. Residual df vary because three of the 44 males lacked orange spots

Dependent variable (units)	Maternal carotenoid diet	Offspring carotenoid diet	Maternal × offspring diet	R^2 adjusted	df
Orange spot carotenoid content (ng)	0·25 ns	9·66**	0·48 ns	0·21	1, 37
Orange spot carotenoid concentration (μg/mm^2)	0·0001 ns	28·98****	0·01 ns	0·43	1, 37
Non-orange skin carotenoid content (ng)	1·72 ns	27·12****	0·68 ns	0·37	1, 40
Non-orange skin carotenoid concentration (μg/mm^2)	1·51 ns	29·97****	1·25 ns	0·39	1, 40
Orange spot drosopterin content (ng)	0·15 ns	1·18 ns	0·17 ns	0·00	1, 37
Orange spot drosopterin concentration (μg/mm^2)	0·01 ns	1·27 ns	0·03 ns	0·00	1, 37
Orange spot area (% of body area)	0·14 ns	2·59 ns	0·64 ns	0·00	1, 40
Orange spot luminance	2·55 ns	0·57 ns	0·00 ns	0·02	1, 37

ns, non-significant: $P > 0·1$, *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, ****$P < 0·0001$.

OFFSPRING SIZE, CONDITION AND GROWTH (EXPERIMENT 2)

There was no significant effect of maternal carotenoid diet on newborn offspring standard length ($n = 33$ litters; $F_{1,28} = 1·18$, $P = 0·28$), mass ($F_{1,26} = 1·20$, $P = 0·28$) or condition ($F_{1,28} = 0·55$, $P = 0·46$) and no significant population by maternal carotenoid diet interaction (all $P \geq 0·5$). Offspring growth rate, whether measured from changes in mass or length, was not significantly affected by the level of carotenoids in the maternal diet ($n = 25$ litters; mass: $F_{1,22} = 0·06$, $P = 0·8$; length: $F_{1,21} = 0·006$, $P = 0·94$) or the offspring diet (mass: $F_{1,22} = 0·076$, $P = 0·78$; length: $F_{1,21} = 0·79$, $P = 0·38$), and there was no significant interaction between maternal and offspring diet (mass: $F_{1,22} = 0·32$, $P = 0·86$; length: $F_{1,21} = 0·01$, $P = 0·92$). The covariates that were retained, their relationship with dependent variables, and their significance levels in the final models were as follows: (i) offspring mass ($P = 0·034$) and condition ($P = 0·027$) were greater in second litters than in first litters; (ii) litter size was negatively associated with newborn offspring mass ($P = 0·026$) and standard length ($P = 0·055$) and offspring growth rate in mass ($P = 0·002$) and length ($P = 0·004$); (iii) dam condition was positively associated with newborn offspring condition ($P = 0·048$); (iv) sire condition was positively associated with offspring growth in length ($P = 0·08$); and (v) dam length was positively associated with newborn offspring mass ($P = 0·03$).

MATURE MALE OFFSPRING SIZE AND CONDITION (EXPERIMENT 2)

We found no significant effect of maternal carotenoid diet or offspring carotenoid diet on the standard length ($N = 44$ males; maternal diet $F_{1,40} = 0·41$, $P = 0·52$; offspring diet $F_{1,40} = 0·15$, $P = 0·70$), body mass (maternal diet $F_{1,39} = 0·63$, $P = 0·43$; offspring diet $F_{1,39} = 0·018$, $P = 0·89$) or condition (maternal diet $F_{1,38} = 0·24$, $P = 0·63$; offspring diet $F_{1,38} = 0·24$, $P = 0·63$) of mature male offspring and no significant interaction between maternal and offspring diet (all $P \geq 0·2$). The condition of mature male offspring was negatively associated with dam length ($P = 0·02$); both male offspring mass and condition were negatively associated with litter number (mass $P = 0·02$; condition $P = 0·002$); no other covariates were retained in the final models. With sub-litters as sampling units, dam length was not a significant covariate ($P = 0·08$) but otherwise the results were the same at an α level of 0·05.

MATURE MALE OFFSPRING PIGMENTATION AND COLOUR (EXPERIMENT 2)

Despite the large effect of maternal carotenoid intake on egg carotenoid content (see above), there was no residual effect on the pigmentation of mature sons (Table 2). If maternal carotenoids had influenced the pigmentation of sons, we expected this effect to be most pronounced for offspring raised on the trace-carotenoid diet, but there was no evidence for such an interaction (Table 2). The level of carotenoids in the diet of male offspring themselves had the expected, positive effect on the amount and concentration of carotenoids in the orange spots and in the skin outside the orange spots (Table 2). Neither maternal nor offspring carotenoid diet affected the drosopterin content of the orange spots, the area of the body covered by any class of colour spots, or the luminance of the colour spots (all $P > 0·1$; only orange spots results are shown in Table 2).

The location of orange spots in colour space was significantly affected by offspring carotenoid diet but not by maternal carotenoid diet (Table 3). Examination of cone excitation contrasts revealed that offspring carotenoid diet primarily influenced the magnitude of contrasts between long-wavelength cones and the other three cone classes (long vs. medium, $t = 4·87$, $P < 0·0001$; long vs. short, $t = 4·04$, $P = 0·0002$; long vs. ultraviolet, $t = 3·44$, $P = 0·0014$; medium vs. short; $t = 2·19$, $P = 0·034$; all other pair-wise cone contrasts, $P > 0·1$). These results are consistent with the absorbance properties of the dominant carotenoid in guppy skin (tunaxanthin, $\lambda_{max} = 438$ nm; Hudon, Grether & Millie 2003) and the λ_{max} values of guppy cones (389, 410, 465 and 543 nm; Archer & Lythgoe 1990), as well as with previous research (Grether 2000, Grether *et al.* 2005a).

Table 3. Effects of the experimental carotenoid diets on the location of mature male offspring colour spots in tetrahedral colour space (Experiment 2). The values shown are proportions of the total squared distance associated with each term in the AOD model. Significance levels are based on randomization tests (n = 41 males)

	Colour spot class		
Effect	Orange	Blue-green	UV-violet
Maternal diet	0·060 ns	0·077 ns	0·041 ns
Offspring diet	0·935**	0·004 ns	0·956 ns
Interaction	0·005 ns	0·919*	0·003 ns

ns, non-significant: $P > 0{\cdot}2$, $*P < 0{\cdot}05$, $**P < 0{\cdot}005$.

The only suggestion of a possible maternal effect on the colouration of mature sons was a significant maternal by offspring diet interaction for blue-green spots (Table 3). Examination of cone excitation contrasts using two-way ANOVA revealed that the interaction involved contrasts between the long-wavelength cone and the other three cone classes (long vs. other, all $P = 0{\cdot}03$; all other cone contrasts, $P > 0{\cdot}05$). Taken at face value, the level of carotenoids in a male's diet reduced long-wavelength cone output relative to that of the other cone classes if his mother was raised on the trace-carotenoid diet but had the reverse effect if his mother was raised on the high-carotenoid diet (not shown). No post-hoc comparisons between diet subgroups were significant (all Bonferroni $P > 0{\cdot}2$), however, and we can think of no biological explanation for such an interaction.

Discussion

We found an intriguing pattern of carotenoid allocation within female guppies. Skin carotenoid content was only weakly affected by the level of carotenoids in the diet while egg carotenoid content was strongly affected. Thus, as carotenoid intake increases, females deposit proportionally more carotenoids into the eggs. This pattern of carotenoid allocation could have evolved to maximize the benefits to be obtained from carotenoids or, alternatively, to dispose of pigments that might otherwise cause problems for females. The former hypothesis would be supported if maternal carotenoids enhanced some aspect of offspring quality. We found no evidence for such benefits, however, despite the large effect of our experimental diets on carotenoid content of the eggs. It remains possible, of course, that maternal carotenoids have positive effects on offspring fitness correlates that we did not measure (e.g. age of first reproduction, swimming performance, sperm motility) or that such effects depend on environmental factors that were absent in our laboratory aquaria (e.g. parasites, predators, food scarcity). To our knowledge, however, this was the most comprehensive experiment to date on maternal carotenoid effects in any species of fish.

In view of the many proposed and documented benefits of these pigments, it might seem ludicrous to suggest that females would ever benefit from disposing of excess carotenoids. Nevertheless, we think this hypothesis merits testing. One plausible negative effect of carotenoid accumulation is interference with crypsis. Guppies have thin skin and translucent muscle – orange spots on one side of the body of males can sometimes be seen from the other side (G.F. Grether, pers. obs.). Thus, even if excess carotenoids were stored below the skin they might interfere with crypsis. A logical first step towards testing this hypothesis would be to determine whether carotenoids build up faster over time in females that are maintained as virgins compared with females that are allowed to reproduce and, if so, whether virgins are more conspicuous to predators. Although virgins produce eggs, unfertilized eggs do not accumulate in or get extruded from the reproductive tract (Reznick 1983).

It might be possible to reject the disposal hypothesis on biochemical grounds. Ingested carotenoids are often metabolized into other types of carotenoids before being deposited in target tissues. For example, most of the carotenoids deposited in the skin of guppies are esters of tunaxanthin, which are produced metabolically from dietary carotenoids (Hudon *et al*. 2003). The use of tunaxanthin can be explained as an adaptation for maximizing the luminance and chroma of the orange spots of males, compared to the alternatives of using unmodified carotenoids or metabolically derived keto-carotenoids (Hudon *et al*. 2003). If egg carotenoids were also produced metabolically, it would be difficult to explain why females expend energy on the conversion unless it benefits the offspring. We did not identify the carotenoids present in the eggs, but based on the wavelength of peak absorbance (λ_{max}) it appears that female guppies deposit ingested carotenoids into the eggs without metabolic conversion. The carotenoids present in the eggs absorb maximally at 446·8 nm, on average, while those present in the experimental diets (lutein, zeaxanthin and β-carotene) absorb maximally at 445–449 nm (by comparison, the carotenoids in female skin absorb maximally at 441·3 nm). A more detailed biochemical analysis will be required to determine whether ingested carotenoids are indeed deposited in the eggs without conversion, but if this is the case, it would be consistent with the hypothesis that females deposit carotenoids in the eggs to dispose of them (we do not mean to imply that such a result would favour this hypothesis over the alternatives).

How strong is the evidence for maternal-carotenoid effects in birds? In domestic chickens (*Gallus gallus*), carotenoid supplementation experiments have shown that maternally derived carotenoids reduce the susceptibility of offspring tissue to peroxidation *in vitro* (Surai & Speake 1998) and that chicks developing from carotenoid-enriched eggs show increased capacity to incorporate ingested carotenoids into several organs (Koutsos *et al*. 2003 and references therein; also see Karadas *et al*. 2005). In a field experiment on black-headed gulls (*Larus fuscus*), carotenoid-supplemented females were more likely to re-lay following clutch removal and produced eggs with higher concentrations of carotenoids than control females (Blount *et al*. 2001, 2004). Barn swallow (*Hirundo rustica*) nestlings from eggs inoculated with

carotenoids (a proxy for maternally derived carotenoids) showed increased acquired immunity (Saino *et al*. 2003). McGraw *et al*. (2005) found that supplementing the diet of captive female zebra finches (*Taeniopygia guttata*) with carotenoids increased egg carotenoid content, reduced egg lipid peroxide content, increased hatching success, increased nestling survival and increased the redness of the beaks of sons after sexual maturity. Carotenoid turnover is probably too high for beak colour to have been influenced directly by carotenoids of maternal origin, but as in chickens, there is evidence that early exposure to carotenoids and other antioxidants influences the ability of zebra finches to assimilate these compounds later in life (Blount *et al*. 2003). McGraw *et al*.'s (2005) results are especially compelling because the carotenoid levels used were within the natural range, the experimental diets were identical except for the carotenoid content, and the offspring were cross-fostered to avoid confounding maternal carotenoid effects with other parental influences.

Numerous carotenoid supplementation experiments have been carried out on commercially valuable fish, but we are only aware of two studies that provide evidence for maternal effects of carotenoid consumption, and both were carried out on yellowtail (*Seriola quinqueradiata*). Verakunpiriya *et al*. (1997) reported that supplementing the diet of female yellowtail with pure astaxanthin increased the hatching and fertilization rates of the eggs and the production of normal larvae up to 30 p.p.m. astanxanthin. At 40 p.p.m. astaxanthin, however, all measures of egg quality dropped below control diet (0 p.p.m.) levels. Agius *et al*. (2001) reported that supplementing the diet of yellowtail with paprika, which is rich in the carotenoids capsanthin and capsorbin, further enhanced egg hatching rate, compared with 30 p.p.m. astaxanthin, and also improved larval survival. Unfortunately, it is difficult to critically evaluate these two reports because diet group means were presented without error variance estimates, samples sizes were low (n = 5–7 broods per diet group) and no statistical significance tests were presented. Similar experiments on this and other marine fishes have failed to detect positive effects of carotenoid supplementation on egg quality (Watanabe & Vassallo-Agius 2003). In a correlative study, Svensson *et al*. (2006) found no relationship between egg carotenoid content and offspring quality in two-spot gobies (*Gobiusculus flavescens*). Watanabe & Vassallo-Agius (2003) suggested that the effects of carotenoids on egg quality in marine fishes are carotenoid-specific and species-specific.

Carotenoids are only likely to influence offspring quality in species that deposit carotenoids in the eggs (Watanabe & Vassallo-Agius 2003). Beyond this, however, it may be too early to speculate on why carotenoid supplementation has positive effects on offspring quality in some species and not others. The negative results (thus far) with guppies and two-spotted gobies, which do deposit carotenoids in the eggs, suggests that it would be unwarranted to assume that an adaptive trade-off necessarily exists between allocation of carotenoids to eggs vs. other tissues (e.g. see Nordeide, Rudolfsen & Egeland 2006). We concur with Badyaev *et al*. (2006) that it remains unclear whether carotenoid deposition into eggs is a passive consequence of carotenoid consumption and lipid metabolism during egg production or part of an active allocation strategy. However, if carotenoid deposition in the eggs was found to be an active process, this would not necessarily favour a specific adaptive hypothesis.

Our results have important implications for mate preference evolution. Recall that guppies of both sexes show innate attraction to orange objects but that the strength of this response is influenced by carotenoid intake only in females. This sex difference would be difficult to explain as a product of selection in a foraging context, unless females benefit more than males do from consuming orange fruits when other sources of carotenoids are scarce (Grether *et al*. 2005b). Thus far, we have found male-specific benefits of carotenoid consumption but no female-specific benefits (Grether *et al*. 2004; Kolluru *et al*. 2006a; this study). By process of elimination, these results strengthen the alternative hypothesis that carotenoid-dependence of the orange attraction sensory bias is a byproduct of selection on females to express stronger preference for high-carotenoid males when carotenoids are scarce in the diet (Grether *et al*. 2005b).

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