

Dietary carotenoid availability influences a male's ability to provide parental care

Thomas W. Pike,^a Jonathan D. Blount,^b Jan Lindström,^a and Neil B. Metcalfe^a

^aDivision of Environmental and Evolutionary Biology, University of Glasgow, Glasgow G12 8QQ, UK and ^bCentre for Ecology and Conservation, School of Biosciences, University of Exeter, Cornwall Campus, Penryn, Cornwall TR10 9EZ, UK

Despite convincing evidence that carotenoid availability can have positive physiological effects, we still lack information on the functional consequences of carotenoid limitation at the behavioral level. Given the role carotenoids play in mitigating oxidative stress produced during physical activity and as immunostimulants, one behavioral function on which they may have a significant impact is an individual's capacity to provide parental care. We tested this hypothesis using three-spined sticklebacks (*Gasterosteus aculeatus*), a species in which males provide obligate and intensive paternal care. Males were fed either high or low (but biologically realistic) levels of carotenoids and monitored throughout incubation, during which we quantified 2 key aspects of parental care: their ability to fan their eggs under normoxic and hypoxic conditions (when both the costs and requirements of fanning increase) and their ability to defend their nest against a simulated conspecific male. High-carotenoid diet males fanned their eggs at a significantly higher rate during hypoxic (but not normoxic) conditions and had higher clutch hatching success than males fed the low-carotenoid diet. There was no evidence that they defended their nest more aggressively. Furthermore, low-carotenoid diet males also appeared to engage in cannibalization of their clutch. These results demonstrate that dietary carotenoid availability can affect a male's ability to provide parental care, and we discuss the potential mechanisms and implications of this finding. *Key words*: carotenoids, oxidative stress, paternal care, reproductive success. [*Behav Ecol* 18:1100–1105 (2007)]

Carotenoid pigments form the basis for much of the yellow, orange, and red sexual ornamentation in the animal kingdom (Goodwin 1984; Olson and Owens 1998), and there is evidence from a number of species that females prefer to mate with carotenoid-rich males (Olson and Owens 1998). One possible explanation for this is that such males are healthier and can provide high-quality direct benefits such as resource-defending ability and parental care (Lozano 1994; von Schantz et al. 1999), both of which can be energetically and physiologically costly (Clutton-Brock 1991). Accordingly, there is correlational evidence that males with more intense carotenoid-based coloration are superior foragers (Hill et al. 2002; Karino et al. 2005) or work harder to provision mates or nestlings (e.g., Hill 1991; Linville et al. 1998; Senar et al. 2002). However, these studies cannot tease apart the effects of carotenoid availability per se on performance from those which are genetically determined. It could be that higher quality individuals can both maintain a higher provisioning rate and signal more. Alternatively, performance may be limited by the availability of carotenoids. One way in which carotenoids could impact on the performance of energetically demanding tasks is by enhancing the capacity of an individual to defend against parasites and diseases (e.g., Blount et al. 2003; McGraw and Ardia 2003; Kolluru et al. 2006; but see Navara and Hill 2003), while in their role as antioxidants, (Krinsky and Yeum 2003; El-Agamey et al. 2004) carotenoids may also protect active muscles against oxidative damage (reviewed in Powers et al. 2004). Working muscle produces radicals and other reactive oxygen species (ROS) that can cause protein oxidation and so contribute to muscle fatigue. These damaging effects can be ameliorated by exogenous dietary antioxidants; indeed, recent evidence suggests that dietary

carotenoid availability is positively associated with flight performance in zebra finches (*Taeniopygia guttata*) (Blount and Matheson 2006). However, no study has manipulated the availability of carotenoids and investigated the effects this has on an individual's capacity to provide parental care.

Three-spined sticklebacks (*Gasterosteus aculeatus*) are an ideal species in which to experimentally test the effects of dietary carotenoid availability on parental care. During the breeding season, male sticklebacks build a nest in which a female deposits her full complement of mature eggs, and all subsequent parental care, which includes defending the nest against territory intruders (including cannibalistic conspecifics) and direct care of the nest and eggs, is by the male (Wootton 1984). Direct care consists of changing nest structure with increasing egg development, removing dead and diseased eggs, and providing the eggs with oxygen and removing waste products by fanning movements of the pectoral fins (Wootton 1976, 1984). Such activities are very expensive in terms of both time and energy (Stanley and Wootton 1986; Chellappa et al. 1989; FitzGerald et al. 1989; Dufresne et al. 1990); fanning, for instance, can consume up to two-thirds of a male's time budget (van Iersel 1953). As a result, males in poor condition may die during the parental phase and thus fail to rear the offspring to independence, or they may eat the eggs in their nest in order to improve their physical condition (Whoriskey and FitzGerald 1985). We have demonstrated previously that male sticklebacks fed a diet that is low in carotenoids suffered from an increased susceptibility to oxidative stress compared with males fed higher levels of carotenoids (Pike et al. 2007). This may limit their ability to maintain an adequate level of paternal care.

In the current experiment, we quantified the investment in parental care by male sticklebacks on 2 diet treatments that differed only in their carotenoid content. We measured the fanning rate under normoxic conditions and also when oxygen levels were experimentally reduced, their ability to defend their nest against conspecific males (which are known to

Address correspondence to T.W. Pike. E-mail: t.pike@bio.gla.ac.uk.
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engage in extra-clutch cannibalism; Wootton 1984), the decline in clutch size over time (which varies greatly between males due to fungal growth on the eggs and cannibalism by the male; Candolin 2000), and the hatching success of the clutch. Male sticklebacks are known to naturally experience fluctuating levels of dissolved oxygen during reproduction (Wootton 1984), and their normal response is to markedly increase their fanning activity (van Iersel 1953; Wootton 1976; Reeb et al. 1984; von Hippel 2000). However, the effectiveness of this compensation is not known. We predicted that the ability to provide paternal care during incubation would be positively associated with dietary carotenoid availability and that an inability to maintain sufficient levels of care would impact negatively on hatching success.

METHODS

Dietary manipulation

Juvenile three-spined sticklebacks were captured with dip nets from the River Endrick, Scotland (56°04'N, 4°23'W) during November 2004. All fish were caught from the same part of the river and so were likely to have experienced similar prior carotenoid availability. Fish were transported to the University of Glasgow on the same day, where they were held in mixed-sex holding aquaria until the start of the breeding season under a simulated natural photoperiod and temperature regime. Throughout the experiment, they were fed to satiation daily on a standardized, custom-made diet made by combining anchovy meal-based fish feed pellets (which are naturally low in carotenoids) with equal quantities of astaxanthin (Carophyll Pink; DSM, Basel, Switzerland) and lutein (FloraGLO; Kemin Health, Des Moines, IA, which also contains approximately 4.2% zeaxanthin) at total concentrations of either 10 µg carotenoids g⁻¹ pellets (low-carotenoid diet) or 200 µg g⁻¹ (high-carotenoid diet). Full details of diet preparation are given elsewhere (Pike et al. 2007). The concentrations of carotenoids used here correspond to the upper and lower limits of the range that has been used to manipulate carotenoid levels in a variety of fish species (Christiansen et al. 1995; Verakunpiriya et al. 1997; Nakano et al. 1999; Grether 2000), and both carotenoid levels are known to allow male sticklebacks to develop sexual coloration and successfully engage in reproductive activities (Pike et al. 2007). Both astaxanthin and lutein are known to occur in this population of stickleback's natural prey (Pike TW, unpublished data from biochemical analysis). Aquaria contained only a water filter and several artificial plants (to provide refuges and reduce stress), and algal growth was controlled by adding 2-chloro-4,6-bis-(ethylamino)-s-triazine (Algae Destroyer, Aquarium Pharmaceuticals, Chalfont, PA). Thus the only source of carotenoids available to the fish was through the feed.

Breeding experiment

Holding tanks were checked daily, and when males began to develop blue eye coloration (an indicator of sexual maturation), they were netted, measured (standard length ± 0.01 mm) and weighed (wet mass ± 0.001 g) (under benzocaine anesthetic), and an index of body condition computed as weight length⁻³ (Wootton 1976). They were then transferred to individual experimental aquaria (33 × 18 × 19 cm), where they were maintained on the same 2 experimental diets as before. The experimental aquaria contained only a filter and an artificial plant and had the same photoperiod, temperature, and water conditions as the holding aquaria. Individual aquaria were separated by opaque partitions so males were not in visual or olfactory contact with each other. When

a sufficient number of males had reached sexual maturation ($n = 40$, 20 from each diet treatment), temperature and photoperiod were held constant (14 ± 3°C, 16:8 h light:dark), and a nesting dish filled with 1 cm sand and around 200 5-cm-long strands of polyester thread as nesting material were added to each aquarium. In order to stimulate nest building, each male was shown a gravid female enclosed in a Plexiglas container for 5 min twice daily for 10 days (e.g., Candolin 2000), after which time all males had completed nest building, developed red nuptial coloration, and entered the courtship phase. The males in each group did not differ significantly in either body weight (mean ± standard error [SE], high-carotenoid males: 0.94 ± 0.06 g, low-carotenoid males: 0.88 ± 0.07 g; $t_{38} = 0.59$, $P = 0.561$), standard length (mean ± SE, high-carotenoid males: 40.9 ± 0.68 mm, low-carotenoid males: 41.9 ± 0.77 mm; $t_{38} = 1.08$, $P = 0.289$), or condition ($t_{38} = 1.14$, $P = 0.260$).

Because we could not manipulate water oxygen levels (see below) in all tanks simultaneously, we staggered the experiment so that one male initiated incubation every 24 h. Each day a male was randomly chosen, with the caveat that we alternated between high- and low-carotenoid diet males, and a gravid female placed in his aquarium (between 0900 and 1100 h) and allowed to spawn. If a female did not spawn within 1 h, she was replaced with a new female. Most females spawned, and the number of females presented to a male did not differ between diet treatments (median [range], high-carotenoid males: 1 [1–3], low-carotenoid males: 1 [1–2]; Mann-Whitney U test: $w = 399.5$, $n_1 = n_2 = 20$, $P = 0.787$). The female was removed immediately after spawning. Pilot studies revealed a strong correlation between the change in female body mass before and after spawning and the mass of eggs laid ($r = 0.99$, $n = 10$, $P < 0.001$; see also Kraak et al. 1997; Candolin 2000). We therefore weighed females (±0.001 g) immediately before and after spawning in order to estimate clutch mass. Females spawn their full complement of eggs at each breeding attempt (Wootton 1984), and there was no significant difference in the mass of clutches spawned in the nests of high- and low-carotenoid diet males (mean ± SE high-carotenoid males: 0.23 ± 0.02 g, low-carotenoid males: 0.21 ± 0.01 g; $t_{38} = 1.10$, $P = 0.278$). Furthermore, because females spawned soon after being placed in a male's tank (generally within 10 min), it is unlikely that they were able to vary any of the eggs' constituents in relation to perceived male quality (Gil et al. 1999; Cunningham and Russell 2000; Pike and Petrie 2005). We do not, therefore, expect that variation in egg quantity or quality could influence our results.

The degree of parental care offered by the male was then quantified by 1) recording the time spent fanning the eggs under normoxic conditions (~95% dissolved oxygen) in three 20-min observation periods spread across the 9-day incubation period; 2) recording the time spent fanning the eggs under hypoxic conditions (30% dissolved oxygen), when the fish are induced to work harder to oxygenate the eggs; 3) measuring the intensity of nest defense (attacks per minute) against a model male intruder presented in a standardized manner; and 4) remeasuring clutch mass on day 8 of incubation (just prior to hatching; Swarup 1958) to determine the percentage clutch reduction and quantify the hatching success of the clutch. Details of each measurement are as follows.

For part 1), the observations were carried out between 0900 and 1100 h on days 1, 3, and 7 of the incubation period while the water in the breeding tank was fully aerated (using compressed air bubbling through an air stone). Fanning behavior was readily observable and easily differentiated from other behaviors such as egg nibbling and nest building (see also Reeb et al. 1984).

Part 2) was carried out on days 5 and 6 of the incubation period. Following earlier studies on fish with similar paternal care (Jones and Reynolds 1999) and nonbreeding sticklebacks (Sneddon and Yerbury 2004), the air stone was removed and the filter turned off and gaseous nitrogen was slowly bubbled into the tank so that the dissolved oxygen content of the water dropped to 30% normoxia over a 6-h period. Dissolved oxygen levels were then held constant ($\pm 2\%$) for 18 h using a solenoid-operated valve on the supply of nitrogen gas operated by a microcontroller connected to an oxygen probe (Loligo Systems, Tjele, Denmark). A 20-min observation of the percentage time spent fanning was carried out between 0900 and 1100 h on day 6, before the nitrogen gas source was removed, and an air stone was added to slowly (over approximately 6 h) restore the tank to its previous normoxic state.

Part 3) was conducted on day 2 of the incubation period. Each male was submitted to a model aggression test (FitzGerald 1993) in which a model male stickleback was placed into the tank 20–25 cm from the experimental male's nest for 1 min, during which the number of bites made by the nesting male were recorded.

To remeasure clutch mass (part 4), the nesting dish was removed from the aquarium, and the nest turned upside down so that the eggs could be removed with forceps. Excess water was dried from the egg mass on absorbent paper and the eggs weighed (± 0.001 g). The percentage clutch reduction (brought about, for instance, by males removing infertile or diseased eggs or cannibalizing their clutch) was calculated in relation to the starting clutch mass. We then removed all the infertile and undeveloped eggs and estimated hatching success by reweighing the egg mass and expressing it as a percentage of the starting egg mass (Candolin 2000). At this stage of development, developed embryos are clearly visible and easy to differentiate from infertile or underdeveloped eggs (Swarup 1958). The eggs would have hatched on day 9 or 10, and the number of developed, healthy eggs present on day 8 is known to agree with the number of eggs actually hatching when a male is allowed to continue to care for the eggs until hatching (Candolin 2000). The weight of an egg may change during development, but this weight loss should be similar for different treatments and so not confound the results.

Ethical note

This work was carried out under license from the UK Home Office. Sticklebacks are tolerant of low oxygen conditions (van Iersel 1953; Reeb et al. 1984; Wootton 1984), which many populations are likely to encounter naturally (Reeb et al. 1984; Sneddon and Yerbury 2004). Males were only exposed to low oxygen levels for approximately 24 h, during which time they were monitored regularly for signs of stress or illness. Overall they behaved normally under hypoxia, continuing to ventilate and care for their eggs, and we could not detect any behavioral signs of stress. There was no evidence for a significant change in weight over the incubation period in either high-carotenoid males (mean \pm SE change in weight: 3.9 ± 11.2 mg; $t = 0.39$, $n = 20$, $P = 0.724$) or low-carotenoid males (mean \pm SE change in weight: 2.0 ± 10.7 mg; $t = 0.19$, $n = 20$, $P = 0.853$), and no males died during the experiment.

Statistical analysis

The change in the time each male spent fanning over the incubation period was analyzed using a repeated-measures analysis of variance, with fanning duration as the repeated

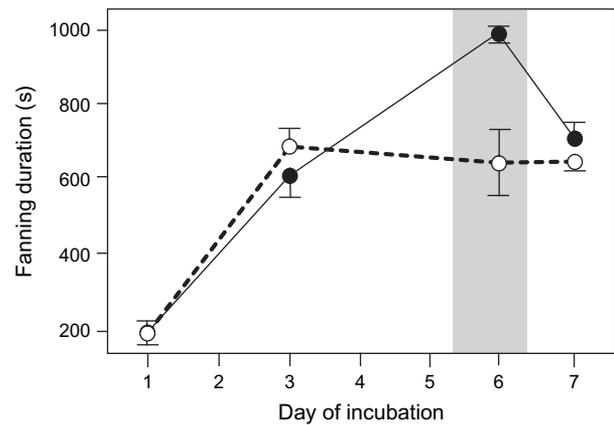


Figure 1

Time spent fanning the eggs (s) over four 20-min observation periods in male sticklebacks fed on high-carotenoid diets (black points, solid lines) and low-carotenoid diets (white points, dashed lines). The shaded area represents the 24-h period when dissolved oxygen levels in the water were reduced to $\sim 30\%$ normoxia.

measure and diet treatment group as a between-subjects factor. Where variables did not meet the assumptions of normality, we used appropriate nonparametric statistics. Means are presented \pm SE and n denotes the sample size.

RESULTS

The time spent fanning changed significantly over the period of paternal care ($F_{3,114} = 89.38$, $P < 0.001$), and there were significant effects of both diet treatment group ($F_{1,38} = 11.36$, $P = 0.002$) and the interaction between diet group and fanning rate ($F_{3,114} = 15.70$, $P < 0.001$; Figure 1). Post hoc tests revealed that there was no difference between diet treatment groups in the time spent fanning during normoxia (Bonferroni-corrected 2-sample t -tests, day 1: $t_{38} = 0.40$, $P = 0.695$; day 3: $t_{38} = 1.11$, $P = 0.273$; day 7: $t_{38} = 1.84$, $P = 0.073$), but males on the high-carotenoid diet fanned significantly more under conditions of hypoxia ($t_{38} = 5.28$, $P < 0.001$); in fact, high-carotenoid males significantly increased their fanning rate during this time compared with before and after (paired t -tests: both $P < 0.001$), whereas low-carotenoid diet males did not ($P = 0.262$ and 0.428 , respectively).

There was no difference between diet treatment groups in the number of attacks on a simulated conspecific intruder (2-sample t -test: $t_{38} = 0.90$, $P = 0.373$). Clutch mass showed a small but significant decline over the incubation period in both diet groups (Wilcoxon signed-rank tests, both $P < 0.001$; Figure 2a). However, there was no significant difference in the absolute (Mann–Whitney U test: $w = 471.0$, $n_1 = n_2 = 20$, $P = 0.102$; Figure 2a) or relative clutch reduction ($w = 352.0$, $n_1 = n_2 = 20$, $P = 0.119$) of low-carotenoid diet males compared with high-carotenoid diet males, although 4 of the low-carotenoid males exhibited a relatively large change in clutch mass (marked as outliers in Figure 2a) which may be due to clutch cannibalism (see Discussion).

All eggs had died prior to hatching in 7 out of the 40 clutches (4 in the low- and 3 in the high-carotenoid diet group; binomial test: $z = 0.42$, $P = 0.677$). Overall, hatching success was significantly higher for males on the high-carotenoid diet (Mann–Whitney U test: $w = 312.5$, $n_1 = n_2 = 20$, $P = 0.009$; Figure 2b), even if the 4 males with a large change in clutch mass are excluded from the analysis ($w = 230.0$, $n_1 = 16$, $n_2 = 20$, $P = 0.037$).

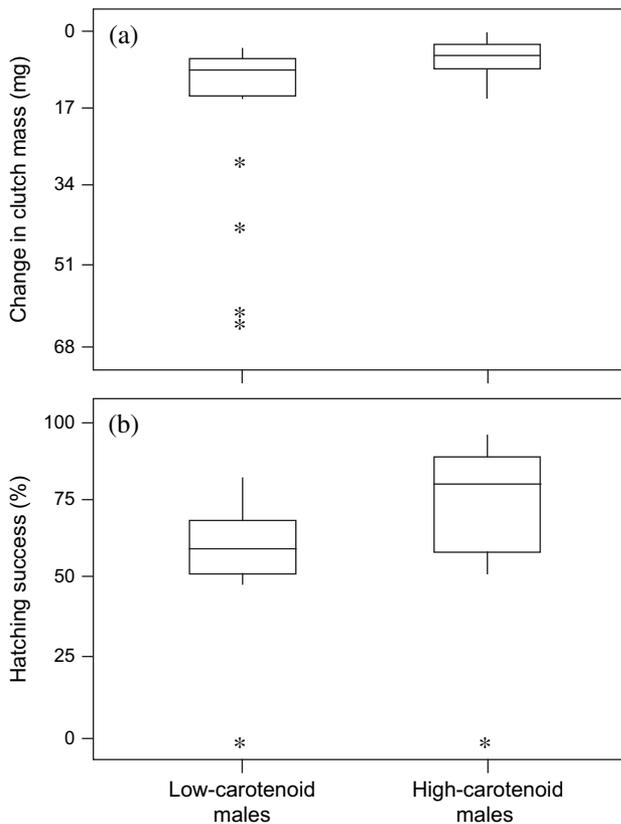


Figure 2
 (a) Reduction in clutch mass over the incubation period and
 (b) hatching success of eggs in the nests of low- and high-carotenoid diet males. Box plots indicate medians, interquartile ranges, maximums, and minimums, with outliers marked by asterisks (*).

DISCUSSION

During normoxic conditions, male sticklebacks in both high- and low-carotenoid diet treatment groups maintained similar levels of fanning activity, and we could find no difference in their aggressive response toward a simulated conspecific intruder. In both groups, the time spent fanning increased as the parental cycle progressed, probably as a result of the increased oxygen requirement of the developing embryos (Davis 1975; Reebbs et al. 1984). On day 6 of the incubation period, when levels of dissolved oxygen were dropped to ~30% normoxia, high-carotenoid diet males increased still further their rate of fanning, presumably to compensate for the lower levels of oxygen in the water. Other studies have also found that a reduction in oxygen levels stimulates an increase in fanning activity in breeding male fish (van Iersel 1953; Reebbs et al. 1984; Torricelli et al. 1985; Jones and Reynolds 1999). However, males on the low-carotenoid diet did not increase their fanning activity and so appeared unable (or unwilling) to compensate for this period of hypoxia. The data suggest that this period of low oxygen did not permanently impair the ability of low-carotenoid males to perform fanning because by day 7 they were matching males on the high-carotenoid diet.

Although sticklebacks can cope with hypoxic conditions (even down to 20% normoxia; Sneddon and Yerbury 2004), such situations are likely to increase overall levels of oxidative stress (Lushchak et al. 2001; Chagas and Val 2006; Lushchak and Bagnyukova 2006). Given the considerable increase in fanning activity apparently needed to oxygenate the eggs dur-

ing hypoxia (see also Jones and Reynolds 1999), existing levels of oxidative stress (which are known to be higher in sticklebacks on low-carotenoid diets; Pike et al. 2007) may have been exacerbated by ROS produced during oxidative metabolism in active muscles, contributing to further oxidative damage and muscle fatigue (McArdle et al. 2001; Powers et al. 2004). It is known that the negative effects of hypoxia-induced oxidative stress can be mitigated with antioxidants (Chagas and Val 2006; Lopez-Olmeda et al. 2006), and so a putative explanation for our results is that a diet containing higher levels of carotenoids enhanced the resistance of muscle tissue to oxidative damage and hence reduced the susceptibility of the muscle to fatigue during the period of increased fanning activity. Low-carotenoid diet males, on the other hand, would have had inadequate antioxidants to cope with increased oxidative stress resulting from both hypoxia and fanning activity, suggesting that they may have been unable to increase fanning activity during hypoxia. It is also possible, however, that the low fanning rate during hypoxia was adaptive. For instance, in the convict cichlid (*Cichlasoma nigrofasciatum*) (Townshend and Wootton 1985) and other populations of three-spined stickleback (Stanley 1983; Östlund and Ahnesjö 1998), parental males on low rations or in poor condition spent less time fanning than those on high rations, and it has been argued that this may allow them to maximize their future reproductive success (Townshend and Wootton 1985). There is also the possibility that carotenoid supplementation, through effects on immune defenses (e.g., Blount et al. 2003; McGraw and Ardia 2003; Kolluru et al. 2006; but see Navara and Hill 2003), could have resulted in fish being less debilitated by parasites or disease and hence more capable of fanning. That low-carotenoid diet males managed to maintain the same fanning level as high-carotenoid males, except during hypoxia, is consistent with a role for current levels of oxidative stress in mediating this behavior.

In a previous study, we demonstrated that female sticklebacks show a significant mating preference for males fed on the high-carotenoid diet over males fed the diet containing lower levels of carotenoids (Pike et al. 2007). We postulated that this was because high-carotenoid diet males had a better chance of surviving a period of energetically expensive incubation and hence successfully rearing offspring to independence. The results of the present study suggest that by selecting males with access to high levels of carotenoids, females may also be choosing males that can provide a better standard of paternal care (although longevity and quality of paternal care are probably not mutually exclusive). In other studies on sticklebacks, paternal fanning was the best predictor of reproductive success (van Iersel 1953; van den Assem 1967; Sargent and Gebler 1980; Östlund and Ahnesjö 1998; von Hippel 2000; but see Stanley 1983). Furthermore, oxygen supply during development can have diverse effects on the subsequent phenotype of offspring. For instance, studies in several species have linked perinatal hypoxia to a reduction in postnatal cognitive abilities (Camm et al. 2001; Simonova et al. 2003; Bachevalier and Vargha-Khadem 2005). Females may therefore gain reproductive benefits by selecting males that can provide adequate oxygenation of their eggs (Östlund and Ahnesjö 1998). The lower hatching success observed in clutches incubated by low-carotenoid diet males may also have been caused by inadequate oxygenation during incubation, particularly during the period of hypoxia when oxygenation would have been especially critical (Jones and Reynolds 1999). However, our data cannot exclude the possibility that the eggs were infertile prior to the onset of incubation, independent of the quality of parental care received. For instance, it is possible that low-carotenoid diet males may have had reduced fertility due to oxidative damage to their sperm, which can

be induced by inadequate access to antioxidants (Wishart 1984; Liu et al. 1997; Surai et al. 1997). There may well have been additional differences in parental care between diet treatments that we did not record; it is possible, for instance, that low-carotenoid diet males were less successful at keeping their eggs free of fungal infection or maintaining their nests.

Within both diet treatment groups, clutch masses declined over the incubation period. This has been observed in other stickleback populations (Candolin 2000) and is likely to be attributable to both a loss of starting resources as metabolic by-products and to the removal of infertile or infected eggs (Whoriskey and FitzGerald 1985). However, there was a dramatic decline in mass in some of the clutches incubated by low-carotenoid diet males, which is consistent with clutch cannibalism (Candolin 2000). This is a relatively common behavior among sticklebacks (Whoriskey and FitzGerald 1985; Candolin 2000) and probably serves as a strategy to recoup investment in current reproduction in order to increase future breeding success (Whoriskey and FitzGerald 1985). It is generally assumed that males eat their eggs in order to increase their energy intake. It is possible, however, that they may be accessing a source of rare or limiting resources, such as carotenoids (Lozano 1994), which are known to be deposited in stickleback eggs (Nordeide et al. 2006). Indeed, because male diets differed only in their carotenoid content and there was no evidence that males in each diet treatment groups differed in body size or condition, the egg cannibalism observed here may have been in order to gain carotenoids. Female mating preference for high-carotenoid diet males (Pike et al. 2007) may thus allow them to gain direct reproductive benefits by choosing males that are unlikely to engage in clutch cannibalism.

In conclusion, the results of this study demonstrate that a male stickleback's ability to provide paternal care can be constrained by his dietary carotenoid intake. Previous studies have tended to concentrate on the physiological effects of carotenoid availability, but these results demonstrate the importance of also addressing the functional consequences of carotenoid limitation at the behavioral level.

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