**Testing the interactive effects of carotenoids and polyunsaturated
fatty acids on ejaculate traits in the guppy *Poecilia reticulata*
(Pisces: Poeciliidae)**

M. M. Rahman^{*}, C. Gasparini^{*}, G. M. Turchini[†] and J. P. Evans^{*†}

^{*} *Centre for Evolutionary Biology, School of Animal Biology, University of Western
Australia, Crawley 6009, WA, Australia.* and [†] *School of Life and Environmental Sciences,
Faculty of Science, Engineering and Built Environment, Deakin University, Warrnambool,
3280, VIC, Australia.*

Running title: Diet and sperm traits in *P. reticulata*

[†] Author to whom correspondence should be addressed. Tel.: +61 8 6488 2010; Fax: +61 8
6488 1029 email: jonathan.evans@uwa.edu.au

1 Using the polyandrous livebearing guppy, *Poecilia reticulata* Peters this study revealed no
2 main effects of carotenoids in the diet on ejaculate traits, but significant main effects of
3 polyunsaturated fatty acids on sperm viability and weak but significant interacting effects of
4 both nutrients on sperm length. Collectively, these findings add evidence that polyunsaturated
5 fatty acids are critical determinants of sperm quality, but also provide tentative evidence that
6 for some traits these effects may be moderated by carotenoid intake.

7
8 Key words: nutrients, condition dependence, oxidative stress, life history, sperm competition

1 Carotenoids continue to attract the attention of evolutionary biologists, primarily because of
2 their antioxidant properties and consequent effects on life history traits (Blount *et al.*, 2001;
3 Svensson & Wong, 2011). Carotenoids are, however, fat-soluble antioxidants and their
4 absorption and bioconversion can be moderated by the intake of other nutrients, including
5 polyunsaturated fatty acids (PUFA) (Castenmiller & West, 1998; Failla *et al.*, 2014).
6 Carotenoids can themselves protect fatty acids from oxidation, particularly omega-3 long-
7 chain PUFA (here termed ‘n3LC-PUFA’) (Wang *et al.*, 2008). Like carotenoids, n3LC-
8 PUFA are important modulators of numerous physiological processes, including sperm
9 production (Wathes *et al.*, 2007; Robbins *et al.*, 2012).

10 Given the expected interacting effects of dietary carotenoids and n3LC-PUFA, it is
11 surprising that until now the effects of both nutrients on ejaculate traits have been studied
12 independently of each other. For example, experimentally induced changes in oxidative stress
13 and dietary carotenoid manipulation have been linked to the expression of ejaculate traits and
14 fertilisation rates in birds and fishes (e.g. Helfenstein *et al.*, 2010; Pike *et al.*, 2010), while
15 n3LC-PUFA regulates sperm quality across several species (Rooke *et al.*, 2001; Wathes *et*
16 *al.*, 2007; Robbins *et al.*, 2012). However, the extent to which n3LC-PUFA and carotenoids
17 interact to determine ejaculate quality has yet to be determined in any species.

18 This study tested for interactive effects of dietary carotenoids and n3LC-PUFA on
19 ejaculate traits in the guppy (*Poecilia reticulata* Peters), a highly polyandrous livebearing
20 freshwater fish. The experiment focussed on traits known to predict reproductive fitness in
21 poeciliid fishes (Boschetto *et al.*, 2011; Smith, 2012) and followed two recent papers on the
22 same population revealing (1) no effect of dietary carotenoid manipulation on ejaculate traits
23 (Rahman *et al.*, 2013) and (2) significant effects of n3LC-PUFA manipulation on sperm
24 viability – the proportion of live sperm in the ejaculate (Rahman *et al.*, 2014). The present
25 experiment extended Rahman *et al.*’s (2013) study by determining whether any effect of

carotenoids on ejaculate traits is either moderated or promoted by n3LC-PUFA. To this end, one hundred and twenty males (aged 3 months) were divided haphazardly into four experimental dietary groups ($n = 30$ for each group; see Table I). The experimental diets differed in the level of carotenoids (hereafter termed ‘carotenoid-enriched’ and ‘carotenoid-reduced’) and n3LC-PUFA (hereafter termed ‘n3LC-enriched’ and ‘n3LC-reduced’), and are described in detail elsewhere (Rahman *et al.*, 2013; Rahman *et al.*, 2014). Carotenoid-enriched diets were compositionally similar to the carotenoid-reduced diets except for the presence of four carotenoid pigments: leutin, zeaxanthin, astaxanthin and β -carotene (Grether, 2000; Kolluru *et al.*, 2006). Within each of the two carotenoid treatments, two n3LC-PUFA diets were formulated – each containing equal levels of total fatty acids but differing in n3LC-PUFA content, which was counterbalanced by increased amounts of oleic acid (a monounsaturated fatty acid) (see Table I). Once assigned to their allotted treatments, males were reared individually in separate 2 l aquaria for three months and fed *ad libitum* the crumbled diet twice daily (six days per week) until they were tested at six months old.

Sperm samples were collected from the anaesthetised males by applying light pressure to the abdomen. From this total sperm pool, two spermatozeugmata (unencapsulated sperm bundles) were collected for sperm velocity estimates and ten spermatozeugmata for sperm viability assays. The remaining sperm bundles were collected for sperm counts and sperm length measures (see below).

Computer-assisted sperm analyses (CASA) were used to estimate sperm velocity using the CEROS sperm tracker (Hamilton-Thorne Research, www.hamiltonthorne.com). The two spermatozeugmata were placed into a single well of a 12-well multitest slide (MP Biomedicals, www.mpbio.com) pre-treated with 1% polyvinyl alcohol (Sigma-Aldrich, www.sigmaaldrich.com) to avoid sperm sticking to the glass slide (Wilson-Leedy & Ingermann, 2007). Sperm velocity was estimated immediately following activation by 150

1 mM KCl with 2 mg ml⁻¹ bovine serum albumin (Sigma-Aldrich, www.sigmaaldrich.com)
2 (Billard & Cosson, 1990). The ensuing measures of average path velocity (VAP; $\mu\text{m s}^{-1}$)
3 were based on an average of 59.05 ± 4.21 SE sperm tracks per sample. A live/dead sperm
4 viability assay (Invitrogen, Molecular Probes) was used to estimate the proportion of live
5 sperm from 200 sperm cells per sample. Sperm counts were estimated using an improved
6 Neubauer haemocytometer and corrected for sperm removed for other assays (Evans, 2009).
7 *ImageJ* was used to measure the total length of sperm from digital photographs of 10 (where
8 possible) undamaged spermatozoa (mean number of sperm cells analysed per male = $9.91 \pm$
9 0.06 SE; range = 5 – 10).

10 One of the $n=120$ fish died during the experiment and four males did not produce
11 sperm (final sample size $n = 115$). Data were checked for normality and transformed as
12 appropriate. A multivariate analysis of variance (MANOVA) was performed to test for an
13 overall effect of the two diet treatments (carotenoid and n3LC-PUFA) and their interaction
14 (carotenoid-by-n3LC-PUFA) on the sperm variables. Separate univariate analyses of variance
15 (ANOVA) models were performed subsequently to detect specific treatment effects on each
16 trait. Treatment groups and their interaction were fitted as fixed effects in all models. All
17 models were run using the ‘car’ package of ‘R’ version 3.1.0 (R Development Core Team,
18 2014).

19 The MANOVA revealed an overall significant effect of n3LC-PUFA on the sperm
20 traits ($F_{4,105} = 4.039$, $P < 0.004$), while carotenoid intake and the interaction between
21 carotenoids and n3LC-PUFA had no significant effects ($F_{4,105} = 0.153$, $P = 0.961$; $F_{4,105} =$
22 1.623 , $P = 0.174$, respectively). The univariate tests confirmed that n3LC-PUFA intake had a
23 significant effect on sperm viability ($F_{1,110} = 12.96$, $P = 0.0005$; see Fig. 1). Interestingly, the
24 univariate tests revealed a weak, but significant, interacting effect of carotenoids and n3LC-

1 PUFA on sperm length ($F_{1,109} = 4.28$, $P < 0.041$). According to this latter model, sperm were
2 significantly longer in the carotenoid-enriched group when n3LC-PUFA levels were reduced.

3 The findings for n3LC-PUFA confirm recent evidence that polyunsaturated fatty acids
4 play an important role in regulating sperm viability in guppies (Rahman *et al.*, 2014), a
5 finding that has also been reported for other taxa (Al-Daraji *et al.*, 2010; Robbins *et al.*,
6 2012). The results from the present study also corroborate recent evidence that carotenoid
7 intake has no overall main effect on ejaculate traits in guppies or cichlids (Rahman *et al.*,
8 2013; Sullivan *et al.*, 2014), although the evidence for weak interacting effects of carotenoids
9 and n3LC-PUFA on sperm length provide tentative evidence that any effect of carotenoids on
10 sperm traits may be contingent on other nutrients (Almbro *et al.*, 2011). Contrary to
11 expectation, however, this study revealed no further evidence that n3LC-PUFA moderate the
12 action of carotenoids on ejaculate traits (see also Marri & Richner, 2014).

13 The lack of any main effect of carotenoids on ejaculate traits in guppies contrasts with
14 prior empirical evidence from other taxa that carotenoids influence sperm motility (e.g.
15 Helfenstein *et al.*, 2010; Tas *et al.*, 2010), sperm morphology (e.g. Atessahin *et al.*, 2006),
16 and both sperm viability and sperm counts (e.g. Mangiagalli *et al.*, 2012; Durairajanayagam
17 *et al.*, 2014). One possible explanation for the present results is that carotenoids are only
18 minor antioxidants in the focal population (see: Costantini & Moller, 2008; Marri & Richner,
19 2014). Further studies are needed to determine whether other nutrients, including vitamins
20 (e.g. vitamin E: Almbro *et al.*, 2011; vitamin E & C: Marri & Richner, 2014) and/or
21 hormones (e.g. Testosterone: Blas *et al.*, 2006), modulate the action of carotenoids on
22 ejaculate traits.

23 The authors thank Cameron Duggin for assistance with maintenance and husbandry,
24 Fernando Norambuena and Sagiv Kolkovski (NutraKol Pty Ltd.) for help with food
25 preparation, and the Australian Research Council (Discovery Grant), European Union

- 1 (Outgoing Marie Curie Fellowship) and the University of Western Australia for funding. This
- 2 work was approved by UWA's Animal Ethics Committee (RA/3/100/513).

Figure 1.

References

- Al-Daraji, H. J., Al-Mashadani, H. A., Al-Hayani, W. K., Al-Hassani, A. S. & Mirza, H. A. (2010). Effect of n-3 and n-6 fatty acid supplemented diets on semen quality in Japanese quail (*Coturnix coturnix japonica*). *International Journal of Poultry Science* **9**, 656-663.
- Almbro, M., Dowling, D. K. & Simmons, L. W. (2011). Effects of vitamin E and beta-carotene on sperm competitiveness. *Ecology Letters* **14**, 891-895.
- Atessahin, A., Karahan, I., Turk, G., Gur, S., Yilmaz, S. & Ceribasi, A. O. (2006). Protective role of lycopene on cisplatin-induced changes in sperm characteristics, testicular damage and oxidative stress in rats. *Reproductive Toxicology* **21**, 42-47.
- Billard, R. & Cosson, M. P. (1990). The energetics of fish sperm motility. In: *Controls of Sperm Motility: Biological and Clinical Aspects* (C. Gagnon, ed), pp. 153–173. CRC Press Inc., Florida.
- Blas, J., Perez-Rodriguez, L., Bortolotti, G. R., Vinuela, J. & Marchant, T. A. (2006). Testosterone increases bioavailability of carotenoids: Insights into the honesty of sexual signaling. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 18633-18637.
- Blount, J. D., Møller, A. P. & Houston, D. C. (2001). Antioxidants, showy males and sperm quality. *Ecology Letters* **4**, 393-396.
- Boschetto, C., Gasparini, C. & Pilastro, A. (2011). Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology* **65**, 813-821.
- Castenmiller, J. J. M. & West, C. E. (1998). Bioavailability and bioconversion of carotenoids. *Annual Review of Nutrition* **18**, 19-38.
- Costantini, D. & Moller, A. P. (2008). Carotenoids are minor antioxidants for birds. *Functional Ecology* **22**, 367-370.
- Durairajanayagam, D., Agarwal, A., Ong, C. & Prashast, P. (2014). Lycopene and male infertility. *Asian Journal of Andrology* **16**, 420-425.
- Evans, J. P. (2009). No evidence for sperm priming responses under varying sperm competition risk or intensity in guppies. *Naturwissenschaften* **96**, 771-779.
- Failla, M. L., Chitchumronchokchai, C., Ferruzzi, M. G., Goltz, S. R. & Campbell, W. W. (2014). Unsaturated fatty acids promote bioaccessibility and basolateral secretion of carotenoids and alpha-tocopherol by Caco-2 cells. *Food & Function* **5**, 1101-1112.

- Grether, G. F. (2000). Carotenoid limitation and mate preference evolution: A test of the indicator hypothesis in guppies (*Poecilia reticulata*). *Evolution* **54**, 1712-1724.
- Helfenstein, F., Losdat, S., Moller, A. P., Blount, J. D. & Richner, H. (2010). Sperm of colourful males are better protected against oxidative stress. *Ecology Letters* **13**, 213-222.
- Kolluru, G. R., Ruiz, N. C., Del Cid, N., Dunlop, E. & Grether, G. F. (2006). The effects of carotenoid and food intake on caudal fin regeneration in male guppies. *Journal of Fish Biology* **68**, 1002-1012.
- Mangiagalli, M. G., Cesari, V., Cerolini, S., Luzi, F. & Toschi, I. (2012). Effect of lycopene supplementation on semen quality and reproductive performance in rabbit. *World Rabbit Science* **20**, 141-148.
- Marri, V. & Richner, H. (2014). Differential effects of vitamins E and C and carotenoids on growth, resistance to oxidative stress, fledging success and plumage colouration in wild great tits. *Journal of Experimental Biology* **217**, 1478-1484.
- Pike, T. W., Blount, J. D., Lindstroem, J. & Metcalfe, N. B. (2010). Dietary carotenoid availability, sexual signalling and functional fertility in sticklebacks. *Biology Letters* **6**, 191-193.
- R Development Core Team (2014). R: a language and environment for statistical computing, version 3.1.0. R Foundation for Statistical Computing, Vienna, Austria. Available: <http://cran.r-project.org/>.
- Rahman, M. M., Kelley, J. L. & Evans, J. P. (2013). Condition-dependent expression of pre- and postcopulatory sexual traits in guppies. *Ecology and Evolution* **3**, 2197-2213.
- Rahman, M. M., Turchini, G. M., Gasparini, C., Norambuena, F. & Evans, J. P. (2014). The expression of pre- and postcopulatory sexually selected traits reflects levels of dietary stress in guppies. *Plos One* **9**, e105856.
- Robbins, W. A., Xun, L., FitzGerald, L. Z., Esguerra, S., Henning, S. M. & Carpenter, C. L. (2012). Walnuts improve semen quality in men consuming a western-style diet: randomized control dietary intervention trial. *Biology of Reproduction* **87**, 1-8.
- Rooke, J. A., Shao, C. C. & Speake, B. K. (2001). Effects of feeding tuna oil on the lipid composition of pig spermatozoa and in vitro characteristics of semen. *Reproduction* **121**, 315-322.
- Smith, C. C. (2012). Opposing effects of sperm viability and velocity on the outcome of sperm competition. *Behavioral Ecology* **23**, 820-826.

- Sullivan, M., Brown, A. & Clotfelter, E. (2014). Dietary carotenoids do not improve motility or antioxidant capacity in cichlid fish sperm. *Fish Physiology and Biochemistry* **40**, 1399-1405.
- Svensson, P. A. & Wong, B. B. M. (2011). Carotenoid-based signals in behavioural ecology: A review. *Behaviour* **148**, 131-189.
- Tas, M., Güney Saruhan, B., Kurt, D., Yokuş, B. & Denli, M. (2010). Protective role of lycopene on aflatoxin B1 induced changes sperm characteristics and testicular damages in rats. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi* **16**, 597-604.
- Wang, W., Shinto, L., Connor, W. E. & Quinn, J. F. (2008). Nutritional biomarkers in Alzheimer's disease: The association between carotenoids, n-3 fatty acids, and dementia severity. *Journal of Alzheimers Disease* **13**, 31-38.
- Wathes, D. C., Abayasekara, D. R. E. & Aitken, R. J. (2007). Polyunsaturated fatty acids in male and female reproduction. *Biology of Reproduction* **77**, 190-201.
- Wilson-Leedy, J. G. & Ingermann, R. L. (2007). Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. *Theriogenology* **67**, 661-672.