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Maternal allocation of carotenoids increases tolerance to bacterial infection in brown trout

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Author contributions
LW, LMC and CW designed the project. LW, LMC, DN and CW sampled the fish, did the *in vitro* fertilizations, and distributed the eggs to 24-well plates. All further manipulations and bacterial infections on the embryos were done by LW and LMC. MH measured embryo length and growth. LM, DO, and VV carried out the chemical analyses, DN wrote the macros that were used by LMC to analyse the skin colouration. LW, LMC and CW performed the statistical analyses and wrote the first version of the manuscript that was then critically revised by all other authors.

Abstract
Life-history theory predicts that iteroparous females allocate their resources differently among different breeding seasons depending on their residual reproductive value. In iteroparous salmonids there is typically much variation in egg size, egg number, and in the compounds that females allocate to their clutch. These compounds include various carotenoids whose functions are not sufficiently understood yet. We sampled 37 female and 35 male brown trout from natural streams, collected their gametes for *in vitro* fertilizations, experimentally produced 185 families in 7 full-factorial breeding blocks, raised the developing embryos singly (n = 2,960), and either sham-treated or infected them with *Pseudomonas fluorescens*. We used female redness (as a measure of carotenoids stored in the skin) and their allocation of carotenoids to clutches to infer maternal strategies. Astaxanthin contents largely determined egg colour. Neither egg weight nor female size was correlated with the content of this carotenoid. However, astaxanthin content was positively correlated with larval growth and with tolerance against *P. fluorescens.* There was a negative correlation between female skin redness and the carotenoid content of their eggs. Although higher astaxanthin contents in the eggs were associated with an improvement of early fitness-related traits, some females appeared not to maximally support their current offspring as revealed by the negative correlation between female red skin colouration and egg carotenoid content. This correlation was not explained by female size and supports the prediction of a maternal trade-off between current and future reproduction.
Key words: Tolerance to infection, bacterial infection, *Pseudomonas fluorescens*, Salmonidae, astaxanthin

Introduction

Carotenoids are lipid-soluble hydrocarbons that are synthesized by plants and some bacteria and fungi (Goodwin 1984). Fish need to obtain them through their diet (van den Berg et al. 2000). Carotenoids might represent essential nutrients or play other important roles, for example, in the immune defence or intra-species signalling (Lozano 2001). Carotenoid-based colourations; *i.e.,* yellow, orange and red, are among the most conspicuous signalling traits and are hence often used in the context of sexual selection (*e.g.,* Olson and Owens 1998; Balshine 2012). Sexual selection theory predicts that such signals reveal genetic or parental quality if they are costly (Johnstone 1997); *i.e.,* if carotenoids are limited or risky to obtain (Blount et al. 2000), if there are constraints in the processing and storing of carotenoids (Olson and Owens 1998; Garner et al. 2010), or if they are required for other physiological functions (*e.g.,* Stephensen 2001; Kolluru et al. 2006). Here, we studied the dual roles of different carotenoids as colour pigments and as components involved in the physiological stress response.

Carotenoids may play at least three beneficial physiological roles: (i) antioxidant activity against damage caused by free radicals (Krinsky and Yeum 2003), (ii) improved pathogen resistance by increasing the production of antibodies and the proliferation of immune cells (Blount et al. 2000; Peters 2007), and (iii) embryonic development (Stephensen 2001; Blomhoff and Blomhoff 2006). In fish, maternally derived carotenoids are therefore assumed to have positive effects on embryo performance and survival (Christiansen and Torrissen 1997; Tyndale et al. 2008; Janhunen et al. 2011), but there are conflicting findings. Some feeding experiments found that high doses of carotenoids either do not increase embryo performance (Tyndale et al. 2008; Brown et al. 2016) or even decrease it (Kolluru et al. 2006; Anbazahan et al. 2014). Such findings may indicate dose-dependent effects (*e.g.,* certain carotenoids being toxic at high concentrations). Moreover, in contexts where carotenoid contents were not artificially increased, *i.e.,* where naturally occurring carotenoids were measured, a positive link between carotenoids content in eggs and later embryo performance could not always be found (Svensson et al. 2006; Wilkins et al. 2017). Accordingly, the role of carotenoids in fish eggs is not solved yet.

Carotenoid-based colours seem to signal genetic quality in many birds (Dale et al. 2015). Carotenoids are then typically immobilized in feathers and hence no more available for physiological use (Thomas et al. 2014). The situation seems different in fish where the skin can be used as a storage organ and carotenoids can be re-allocated over time (Garner et al. 2010; Brown et al. 2014). In fishes with parental care, carotenoid-based colouration has been described to signal parental quality, as for example in the three-spined stickleback (*Gasterosteus aculeatus*; Candolin and Tukiainen 2015; Kim and Velando 2016). However, in fishes where males offer no parental care, the role of carotenoid-based signals is less clear. While in some species carotenoid-based colouration is linked to male quality; *e.g.,* in guppies (*Poecilia reticulata*; Grether 2000), in other species conspicuously orange or red coloured males were of average genetic quality (Janhunen et al. 2011) or even below average (Wedekind et al. 2008; Backström et al. 2015).

The functional significance of red colouration in females seems even less clear. In species with paternal care, the evolution of female ornamentation may be a consequence of male choice (Balshine 2012). Indeed, female sticklebacks show carotenoid-based colours on their pelvic spines during the breeding season, and less intense colours after breeding (Nordeide et al. 2013; Amundsen et al. 2015), and female two-spotted goby (*Gobiusculus flavescens*) signal the colour of their eggs

through transparent skin (Svensson et al. 2009). In species with no parental care, we would expect male mate choice to play a limited role (Kraaijeveld et al. 2007). However, females of many species have conspicuous colours, for example in Arctic charr (Janhunen et al. 2011) and in different *Oncorhynchus* species, where carotenoid reserves are mobilized predominantly to eggs but also to skin shortly before the breeding season (Foote et al. 2004; Garner et al. 2010). The functional significance of such female skin colours is still unclear.

Salmonids are an excellent model to study female reproductive strategies. They show no parental care; *i.e.,* there is no differential investment after mating (Sheldon 2000; Balshine 2012). *In vitro* fertilization can be used to produce maternal and paternal half-siblings that allow separating dam and sire effects on offspring performance (Lynch and Walsh 1998). Females produce large numbers of eggs, and embryos can be raised singly under experimental conditions until hatching; *i.e.,* large numbers of replicates allow for full-factorial experimental designs and the study of important interaction terms. Based on these powerful experimental protocols, it could be established that there is significant additive genetic variance for fitness-related traits like tolerance to infection (von Siebenthal et al. 2009; Aykanat et al. 2012), to organic pollution (Jacob et al. 2010; Wilkins et al. 2016), or to chemical pollution (Brazzola et al. 2014). The importance of additive genetic effects increases during embryogenesis and the embryo's own genetics becomes more relevant at later stages of development (Clark et al. 2014). At late embryonic stages, tolerance to infection could even be linked to specific immune genes (Wedekind et al. 2004; Clark et al. 2013).

Dam effects are a mixture of maternal genetic and maternal environmental effects, which together represent the components that females put into their eggs before spawning. Dam effects are usually stronger than sire effects (von Siebenthal et al. 2009; Aykanat et al. 2012) suggesting that maternal environmental effects play a significant role in embryo stress tolerance (Nagler et al. 2000). Variance in maternal environmental effects may reveal differences in female quality (health and vigour) or, in iteroparous salmonids, this variance could result from trade-offs between current reproduction and survival to the next reproductive season (Marshall et al. 2008; Nordeide et al. 2013). Maternally-derived carotenoids in eggs have shown to contribute to overall maternal environmental effects on embryo performance (Wilkins et al. 2017). Hence, they may reveal such female trade-offs.

In this study, we sampled wild brown trout, determined the variation in carotenoid contents of their eggs, and linked these contents to skin colouration (as measure of carotenoid content; Steven 1949) and other female traits. We then experimentally fertilized these eggs in full-factorial breeding blocks to separate maternal from paternal effects, and raised the embryos singly until hatching. Embryos were either exposed to *Pseudomonas fluorescens*, an opportunistic pathogen that has been found on naturally-spawned brown trout eggs in the study area (Wilkins et al. 2015), or they were sham-treated in order to investigate the link between maternally-derived carotenoids and embryo tolerance to infection.

Material and methods

Experimental protocol

Adult brown trout were caught with electrofishing from their natural spawning grounds in a river network of two adjacent tributaries (Kiese and Rotache; Stelkens et al. 2012) of the river Aare in Switzerland. Fish were kept at the *Fischereistützpunkt Reutigen* (a cantonal hatchery) until they were stripped for their gametes. Each female

was then photographed under standardized conditions (Fig. 1) with a colour scale (Stufengraukeil und Farbkarte #13, B.I.G. Photo Equipment) to measure her redness (for its estimation see below). Four eggs per female were frozen in liquid nitrogen for later quantitation of their astaxanthin, capsanthin, lutein, and zeaxanthin content. These eggs were protected from light and stored at -80° C. The remaining gametes were used for full-factorial *in vitro* fertilizations following the protocols described in Jacob et al. (2010). After two hours of egg hardening, standardized photos were taken for egg counting and measurements of egg colours. We produced five different breeding blocks consisting of five females crossed with five males each for the river Kiese (*i.e.,* 5 x 25 families) and two blocks consisting of six females crossed with five males each for the river Rotache (*i.e.,* 2 x 30 families). In total, we used the gametes of 35 males and 37 females to produce 185 families. For every family, 16 newly fertilized eggs were individually distributed to 24-well plates (Falcon, BD Biosciences, Allschwil, Switzerland) in 2 ml of standard water according to OECD guidelines (1992). Standard water had been autoclaved, temperated, and oxygenated before use. Embryos were raised at 6.5° C in a climate chamber with a 12-hour light-dark cycle.

Three breeding blocks of the river Kiese and one breeding block of the river Rotache were either exposed to *P. fluorescens* (PF) at a concentration of 10^6 bacterial cells/ml per well or sham exposed (with standard water only) 18 days after fertilization (75 and 30 families, respectively). The remaining breeding blocks were exposed or sham-exposed 49 days after fertilization (50 and 30 families, respectively). Every family was exposed to both treatments: 8 embryos per family were exposed to the pathogen and the remaining 8 embryos were sham exposed each. We treated embryos at two different time points because the virulence of a bacterial pathogen can depend on embryo development time (Clark et al. 2014). PF cultures were prepared and diluted as described for "PF 1" in Clark et al. (2013).

Embryos were daily monitored for mortality and time until hatching. At the day of hatching, embryos were individually transferred to 12-well plates (Falcon, BD Biosciences, Allschwil, Switzerland) filled with 3 ml of fresh standard water. Plates were then scanned under standardized conditions (Scanner Perfection V37; Epson, Suwa, Japan). This allowed us to individually measure embryonic length and to estimate yolk sac volume at the day of hatching, as in Jensen et al. (2008). Fourteen days after hatching, embryos were scanned again in order to determine each embryo's individual growth rate.

Image analyses

Embryo images were analysed with ImageJ v.1.49u (Schneider et al. 2012). Female standard length, carotenoid-based colouration, and egg redness were also determined in ImageJ. For fish carotenoid-based colouration an ImageJ macro was developed (see details in Electronic Supplementary Material). Briefly, the area of each red spot present on both sides of the female body was measured in pixels. Then, the area of all red spots was summed and divided by the total body area to obtain the proportion of red area. The mean redness of these red spots was estimated as the a* component in the Lab colour space. The Lab colour space contains all perceivable colours, where L represents lightness, and a* and b* are the colour opponent dimensions (a* ranges from green (negative values) to red (positive values), and b* from blue to yellow). Then, the redness values were standardized according to luminosity in each picture. Finally, the redness of the red spots was calculated relative to the redness value of the

red reference of the colour scale present in the pictures (*i.e.,* relative redness). Egg redness was determined analogously on 10 eggs per female.

Carotenoid extractions

Eggs were thawed, dried, and weighed before carotenoid extractions. Four eggs were pooled for each of the 37 females, however, two pools got accidentally mixed during handling (final number of pools = 35). Eggs were homogenized with five tungsten beads (3 mm; Qiagen, Hombrechtikon, Switzerland) in a mixer mill (MM300; Retsch, Düsseldorf, Germany) for 2 runs of 2 min at 30 Hz. The homogenate was centrifuged at 20,238 RCF for 2.5 min (Centrifuge 5424; Eppendorf, Hamburg, Germany). The supernatant was kept on ice and protected from light. The pellet went through a second step of bead beating with the residual tungsten beads and 1.2 ml of fresh ethyl acetate. After combining both supernatants, they were dried in a centrifugal evaporator (Centrivap; Labconco, Kansas City, USA) for 70 min with the centrifuge kept at 35° C. Dried carotenoids were kept at -80° C in the dark until quantitation.

Quantitative analysis of carotenoids by UHPLC-ESI-HRMS

Quantitative analyses were conducted on a Xevo G2-S QTOF mass spectrometer coupled to the Acquity UPLC Class Binary Solvent Manager and BTN Sample Manager (Waters, Corporation, Milford, MA). The separation was achieved using an ACQUITY UPLC® BEH C18 1.7 μm column, 2.1 mm x 50 mm (Waters) heated at 35° C. We focused on astaxanthin, lutein and zeaxanthin because these three out of eight carotenoids were found above detection threshold in previous samples taken within the larger study area (Wilkins et al. 2017). Since preliminary analyses also suggested high levels of capsanthin, we therefore included this carotenoid type in our study. Mobile phase consisted of ACN:MeOH 7:3 (v/v) as eluent A and water as eluent B. The separation was carried out at 0.4 mL/min over a 15 min total run time using the following program (Rivera et al. 2014): 0-2 min, isocratic 20% B; 2-3 min, 20-0% B; 3-7 min, isocratic 0% B; 7-8 min, isocratic 0% B and flow increase to 0.6 mL/min; 8-11.6 min, 0% B at 0.6 mL/min; 11.6-12.6 min, 0-20% B at 0.4 mL/min; 12.6-15 min, re-equilibration. The sample manager system temperature was maintained at 10 °C and the injection volume was 2 μL. Mass spectrometer detection was operated in positive ionization using the ZSpray™ dual-orthogonal multimode ESI/APCI/ESCi® source. The TOF mass spectra were acquired in the sensitive mode over the range of *m/z* 300-800 at an acquisition rate of 0.036 sec/spectra. The TOF analyser was calibrated using a solution of sodium formate (0.01 mg/L in isopropanol/H_2O 90:10). A mass accuracy better than 10 ppm was achieved using a leucine-encephalin solution as lock-mass (200 pg/μL in ACN/H_2O (50:50)) infused continuously using the LockSpray source. Source settings were as follows: cone, 25V; capillary, 3 kV, source temperature, 150° C; desolvation temperature, 500° C, cone gas, 10 L/h, desolvation gas, 500 L/h. Data were processed using MassLynx™ 4.1 software and QuanLynx application for quantification. Standard stock solutions of astaxanthin, capsanthin, lutein, and zeaxanthin were prepared at a concentration of 1 mM in DMSO and in Waters® Amber Glass 12x32 mm Screw Neck Vials. Carotenoid solutions were further diluted 1:1000 in ACN:MeOH 7:3 (v/v) and calibration curves achieved by a serial dilution in the 1–50 nM concentration range (Supplementary Fig. S1). Egg samples were diluted 1:250 in ACN:MeOH 7:3 (v/v) before LC-MS analysis in order to fit into the calibration curves. Extracted ions chromatograms (XIC) were based on a retention time (RT) window of ±0.5 min with a mass-extraction-window (MEW) of ±25 ppm centred on m/z_{theor} of each carotenoid.

The average peak area of three replicate injections at each concentration was used for each data point. Calibration curves were fitted with a polynomial order 2 equation with $R^2 > 0.995$ for all carotenoids.

Statistical analyses

The relationships between the contents of the four egg carotenoid types studied here were analysed with Pearson's product moment correlations (r) after graphical inspection of the data suggested that the model assumptions were not significantly violated. Analogously, the links between female traits (length, relative redness and proportional red area) and astaxanthin, capsanthin, lutein, and zeaxanthin egg contents were also analysed with Pearson's product moment correlations. The total amount of carotenoids that females allocated to their clutch was log transformed to meet the assumption of normality. In order to test whether timing of infection had an effect on the virulence of *P. fluorescens*, mean embryo responses between early and late infections were compared using Wilcoxon rank tests for survival rates and paired t-tests for time until hatching, length at hatching, yolk sac volume at hatching, length 14 days after hatching, and larval growth.

For the remaining analyses, embryo survival (dead before hatching or hatched) was analysed in generalized linear mixed models, while timing of hatching (days), length at hatching (mm), yolk sac volume at hatching (mm^3), and growth were analysed as continuous response variables in linear mixed models (LMM). For each of these models, treatment (control or PF), mean egg weight (g) and total carotenoid content per egg (astaxanthin or lutein in nM/L) were entered as fixed effects, while dam was entered as a random effect. Our full-factorial breeding design controls for potential sire effects that could therefore be ignored here. The interaction terms of treatment with dam, egg weight and carotenoid content per egg were also investigated. Analogous models were analysed with carotenoid concentrations (astaxanthin and lutein) per gram of egg instead of total content in each egg. Capsanthin and zeaxanthin were not included in the models because (i) these carotenoids were significantly correlated to astaxanthin content, and (ii) capsanthin could not be quantified above detection limit in seven out of 35 females.

To test the significance of an effect, a model including or lacking the term of interest was compared to the reference model. The goodness of fit of the different models is given by the logarithm of the approximated likelihood and by the Akaike information criterion. To test if models differ in their goodness of fit, the models were compared with likelihood ratio tests (LRT). When there was a strong *a priori* expectancy about the direction of an effect, the significance level of the p-value obtained from the LRT test was adjusted accordingly (Rice and Gaines 1994), i.e. p value had to be ≤ 0.01 to be considered significant for correlations in the unexpected direction. All statistical analyses were performed in R v.3.1.3 (R Development Core Team 2015), and mixed effect models were run with the lme4 package v.1.1.11 (Bates and Sarkar 2007).

Results

Quantification of carotenoids

All four carotenoids could be separated and quantified using the described UPLC-ESI-HRMS method that provided a good inter- and intra-assay reproducibility in retention time (RSD = 0.1%). The control of the sample temperature turned out to be a crucial parameter to achieve this performance. In our conditions, electrospray ionization (ESI) tended to be more sensitive compared to atmospheric pressure chemical ionization (APCI), and the molecular ion species observed was either the

protonated molecule $[M+H]^+$ (astaxanthin), the molecular ion $[M]^{+\cdot}$ (lutein and zeaxanthin) or both (capsanthin). Figure S2 shows the extracted ion chromatograms (XIC) of the four carotenoids together with their corresponding HR-MS spectra resulting in a mass accuracy < 10 ppm for all of them. Despite their similarities in structure, lutein and zeaxanthin could still be separated and quantified using those conditions (Supplementary Table S1). The limit of detection (LOD, defined as S/N > 3) and limit of quantitation (LOQ, defined as S/N > 10) were 1 nM for lutein and zeaxanthin, and 3 nM for capsanthin and astaxanthin. Replicate injections resulted in an average % CV (Coefficient of Variation) within sample of 18% for lutein, 10% for zeaxanthin and astaxanthin, and 12% for capsanthin.

Maternal traits

Astaxanthin, lutein and zeaxanthin were found in the eggs of all 35 females in the following amounts (means ± 95% confidence intervals): astaxanthin (720 nM/egg ± 165), lutein (178 nM/egg ± 27) and zeaxanthin (848 nM/egg ± 129). Capsanthin could only be detected above threshold level in 28 females and showed wide confidence intervals (700 nM/egg ± 327). Because capsanthin content was highly variable and at a different scale than the other measured carotenoids, correlations involving capsanthin content were displayed in independent figures in the Electronic Supplementary Material.

The contents of astaxanthin and zeaxanthin in the eggs were correlated (r = 0.39, n = 35, $p = 0.019$). The respective correlations to lutein were not significant (lutein *vs*. astaxanthin: r = 0.11, $p = 0.52$; lutein *vs*. zeaxanthin: r = 0.31, $p = 0.07$). Capsanthin was correlated to astaxanthin (r = 0.36, $p = 0.03$), lutein (r = 0.41, $p = 0.01$) and zeaxanthin (r = 0.57, $p < 0.001$). When comparing female traits to egg carotenoid content (Fig. 2A – D & S3A – D), we found that astaxanthin and zeaxanthin always behaved similarly. Astaxanthin, capsanthin and zeaxanthin could not be significantly predicted by female body length (Fig. 2A & S3A; astaxanthin: r = -0.06, $p = 0.71$; capsanthin: r = 0.15, $p = 0.38$; zeaxanthin: r = 0.05, $p = 0.76$). Contrarily, lutein was negatively linked to female length (Fig. 2A; r = -0.40, $p = 0.02$). None of the four carotenoids was correlated to egg weight (Fig. 2B & S3B; astaxanthin: r = -0.14, $p = 0.43$; capsanthin: r = 0.07, $p = 0.65$; zeaxanthin: r = 0.09, $p = 0.60$; lutein: r = -0.20, $p = 0.26$).

The proportional area of red coloured skin of the mother was negatively correlated to astaxanthin and zeaxanthin content per egg (Fig. 2C; astaxanthin: r = -0.40, $p = 0.016$; zeaxanthin: r = -0.53, $p = 0.001$) but not to capsanthin (Fig. S3C; r = -0.15, $p = 0.38$) or lutein (Fig. 2C; r = -0.22, $p = 0.21$). The total amount of each astaxanthin, lutein, and zeaxanthin that was provided to the total clutch was negatively correlated to the proportional area of red coloured skin (Fig. 2D; astaxanthin: r = -0.48, $p = 0.004$; lutein: r = -0.42, $p = 0.012$; zeaxanthin: r = -0.58, $p < 0.001$). The total amount of capsanthin provided to the clutch was, however, not correlated to the proportional area of red coloured skin (Fig. S3D; r = -0.16, $p = 0.36$). Skin relative redness was not correlated to the carotenoid content of the eggs (absolute r always < 0.21, p always > 0.24). Astaxanthin but not capsanthin, lutein, or zeaxanthin was positively correlated to egg redness (Fig. S4 & S5; astaxanthin: r = 0.54, $p < 0.01$; capsanthin: r = 0.32, $p = 0.06$; lutein: r = -0.09 $p = 0.61$; zeaxanthin: r = 0.17, $p = 0.34$). Female length correlated positively with the weight of the eggs (Fig. S6; r = 0.52, $p = 0.002$). Clutch size did not correlate with female redness measures (*i.e.*, proportional area of red coloured skin: $p = 0.11$, r = -0.27 and skin relative redness: $p = 0.62$, r = -0.08). Since astaxanthin, capsanthin and zeaxanthin

were correlated, our statistical models on embryo viability include only astaxanthin and lutein as independent variables to avoid colinearity problems for further analyses.

Time point of infection

Early infection led to higher mortality than late infection (Wilcoxon $W = 100$, $p = 0.04$) but both mortalities were low (Fig. S7A). All other embryo traits (timing of hatching, length at hatching, yolk sac volume at hatching, and growth) seemed not affected by the timing of infection (Fig. S7B – E; absolute t always < 1.95, p always > 0.06). Therefore, time point was excluded from further analyses.

The effects of egg carotenoids and weight across treatments

Lutein egg content was not significantly linked to any of the analysed embryo traits under pathogen exposure or sham treatment (Table 1 – 3). Astaxanthin content in the eggs did not seem to affect survival (Fig. 3A, Table 1), hatching time (Table 2A) and growth before hatching (Fig. 3B – D, Table 2B – C). However, astaxanthin content was positively correlated with growth after hatching (Fig. 3E, Table 3); *i.e.*, females that allocated more astaxanthin to their eggs produced offspring of faster growth.

Astaxanthin content in the eggs did not seem to affect treatment-linked mortality (Fig. 3F, Table 1). However, it mitigated the effects of *P. fluorescens* on developmental rate and growth before hatching (Fig. 3G – I); *i.e.,* while *P. fluorescens* decreased developmental rate, this negative effect was significantly less pronounced at higher astaxanthin concentrations. Furthermore, there was also a close to significant negative correlation between astaxanthin content in the eggs and differential larval growth between treatments; *i.e.,* growth of pathogen-exposed embryos minus growth of sham-treated embryos (Fig. 3J, Table 3). Analogous analyses on egg astaxanthin and lutein concentrations instead of their total content in the eggs revealed similar results (Table S2 – S4). The only exception was that the correlation between the difference in larval growth between treatments and the astaxanthin concentration was significantly negative (Table S4).

Embryo survival in the control group seemed negatively correlated to egg weight if tested two-tailed (Table 1, Fig. 4A). However, our *a priori* expectancy was that egg size should have positive effects on embryo survival (as repeatedly found before; Einum and Fleming 1999; Einum and Fleming 2000). We therefore adjusted the p value for effects in the unexpected direction (*i.e.,* $p = 0.01$; Rice and Gaines 1994) and conclude that egg size has no significant effect of embryo survival in our study. Embryos of larger eggs hatched earlier (Fig. 4B, Table 2A) but there was no correlation between egg weight and either hatchling length (Fig. 4C, Table 2B) or growth after hatching (Fig. 4E, Table 3). However, yolk sac volume at hatching was positively correlated with egg weight (Fig. 4D, Table 2C). The correlations between embryo survival and egg weight were similar in the infected and non-infected groups (Fig. 4A and F, Table 1). Infected embryos hatched later and this effect increased with egg weight (Fig. 4G, Table 2A). Infected hatchlings were significantly smaller than controls (Fig. 4C and H, Table 2B). Yolk sac volume was not significantly affected by either treatment or egg weight (Fig. 4D and I, Table 2C). If tested two-tailed, infected larvae seemed to grow faster after hatching than uninfected larvae, especially so in heavier eggs (Fig. 4J, Table 3). However, the direction of this effects was again against our *a priori* expectancy (*P. fluorescens* have repeatedly been used in infection experiments and never found to improve embryo performance; von Siebenthal et al. 2009, Clark et al. 2013; Clark et al. 2014), and after adjusting the p value to 0.01 for

effects in the unexpected direction (Rice and Gaines 1994) we conclude that infection had no significant effect on larval growth after hatching.

Discussion

Carotenoid content in the eggs

We found astaxanthin, lutein and zeaxanthin in all eggs, and capsanthin in 80% of our samples. The first three carotenoids have also been found in the eggs of other salmonids (Palace and Werner 2006; Tyndale et al. 2008; Garner et al. 2010). Capsanthin is usually not targeted in salmonid eggs (Tyndale et al. 2008; Garner et al. 2010). Here, it was highly variable and correlated to all the other carotenoids; *i.e.,* when carotenoid concentrations were high, capsanthin concentrations were also elevated. The physiological relevance of capsanthin in fish tissues has not been much studied yet. This is different for the other three xanthophylls, particularly for astaxanthin. It has been shown in rainbow trout that astaxanthin was converted to zeaxanthin during the process of lipid biosynthesis (Schiedt et al. 1986). This could explain why we found these two carotenoids to be highly correlated across females. Lutein was not correlated to astaxanthin or zeaxanthin.

Egg redness was correlated with astaxanthin content as found in previous studies (Tyndale et al. 2008; Garner et al. 2010). Such red colouration has been found to result from conjugated double bonds at the center of the astaxanthin molecule (Ambati et al. 2014). Lutein content was not linked to egg redness. Similar results were found in three-spined sticklebacks where red skin colouration could be linked to astaxanthin content and yellow skin colouration to lutein content (Wedekind et al. 1998). Egg weight did not predict carotenoid content. Under sham-treatment conditions; *i.e.,* when embryos were not challenged, none of the carotenoids influenced embryo development. However, larval growth after hatching was enhanced with increased astaxanthin contents in the eggs. This correlation could not be explained by egg weight or female size. A positive relationship between carotenoid content and larval growth supports the prediction of Blount, Houston and Møller (2000) that the risk of peroxidation ultimately peaks after hatching because of the rapid exposure to high concentrations of atmospheric oxygen. Moreover, with the onset of respiration after hatching, larvae embark on a new phase of high metabolism with an associated risk of oxidative stress during post-hatching growth (Blomhoff and Blomhoff 2006).

Treatment effects

The opportunistic fish pathogen *P. fluorescens* proved to be weakly virulent, in line with previous studies (von Siebenthal et al. 2009; Clark et al. 2013; Clark et al. 2014). Here it had little effect on survival (it decreased survival by only a few percent) and mainly slowed down embryo development until hatching, a stress response that has been observed before in other contexts (Barry et al. 1995; Clark et al. 2013; Clark et al. 2014). The hatchlings of the exposed group were smaller but with similar yolk sac volumes.

Carotenoid effects during pathogen stress

There were no correlations between lutein and embryo or larval development, neither under the stressed nor under the non-stressed condition. We only found a correlation between lutein content in the egg and female size, as well as female skin red colouration, that both remain to be further studied.

Astaxanthin seemed to have no effect on hatchling length and yolk sac volume at hatching, neither in the sham treatment nor under pathogen conditions. However, the more astaxanthin in the eggs, the shorter the delay in hatching time that was caused by our infection protocol. Hence, the virulence of *P. fluorescens* turned out to be mitigated by astaxanthin. Moreover, bacterial infection increases oxidative stress in embryos (Anbazahan et al. 2014), leading to a depletion of antioxidant substances (Stephensen 2001). Astaxanthin had an overall positive effect on growth after hatching that was indeed less pronounced in larvae that had been infected during embryogenesis (this effect was significant when tested with astaxanthin concentrations and close to significance with total content in the eggs).

In the aquatic environment astaxanthin is biosynthesized by microalgae and phytoplankton (Seabra and Pedrosa 2010; Yuan et al. 2011) and it has been cited as the most common carotenoid in fish, as well as in aquatic crustaceans (Seabra and Pedrosa 2010). Although astaxanthin does not possess a pro-vitamin A activity (Yuan et al. 2011), it has been demonstrated that it had the highest antioxidant activity of several naturally occurring carotenoids in humans and birds (Ambati et al. 2014). The great antioxidant potential of astaxanthin results from conjugated double bonds that react with free radicals at the center of this molecule. The lipid-soluble part at the tail of the molecule can link with the cells from the inside to the outside and terminate the detrimental free radical chain reaction in a wide variety of living organisms (Kim et al. 2009). Lutein and zeaxanthin are stereoisomers of each other (Nwachukwu et al. 2016) that have also shown to act as potent antioxidants, however, about ten times less efficient than astaxanthin (Yuan et al. 2011; Ambati et al. 2014). Because astaxanthin was significantly linked to embryo performance and lutein was not, our results are in line with these physiological descriptions of naturally occurring carotenoids and underline the prominent role of astaxanthin during the development of fish embryos.

Female skin colour

Carotenoids are largely responsible for the yellow, orange, and red colours of fishes (recent examples include Pham et al. 2014; Alishahi et al. 2015; Pailan et al. 2015; Yi et al. 2015; Brown et al. 2016; Sorensen et al. 2016). Different colour qualities can reveal the relative contributions of different carotenoids at different stages during life history (Wedekind et al. 1998; Black et al. 2014). The expected links between carotenoids and skin colours has also been found in various salmonids (Bjerkeng et al. 1992; Garner et al. 2010; Backström et al. 2014; Backström et al. 2015) who can show much phenotypic plasticity in their skin colorations (Westley et al. 2013).

In the present study, females differed in red colouration irrespective of their body size, clutch size, or average egg weight. The proportion of red skin relative to total skin area was significantly negatively correlated to the amount of astaxanthin, zeaxanthin and lutein that the females allocated to their clutch and to the content of each of these carotenoids per egg. Hence, the more colourful females produced offspring of lower viability, confirming a previous finding in Arctic charr (Janhunen et al. 2011). We therefore expect redder females to have a higher survival probability to the next breeding season and conclude that female redness does not signal egg quality in brown trout. It remains to be tested whether skin redness is used for other kinds of intra-species signalling (Foote et al. 2004), crypsis (Donnelly and Dill 1984), or for the protection against reactive oxygen species (Tyndale et al. 2008). Our findings highlight the importance of maternally-derived carotenoids on offspring viability and suggest maternal trade-offs between current and future reproduction.

Data accessibility
Data on embryo performance (survival rates, hatching time, length and growth measurements), on paternal characteristics (size, carotenoid concentrations and redness measures), and R-scripts are deposited on the Dryad repository doi: 10.5061/dryad.sj416.

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References
Alishahi M, Karamifar M, Mesbah M (2015) Effects of astaxanthin and Dunaliella salina on skin carotenoids, growth performance and immune response of *Astronotus ocellatus*. Aquaculture International 23: 1239-1248. doi: 10.1007/s10499-015-9880-0

Ambati RR, Phang SM, Ravi S, Aswathanarayana RG (2014) Astaxanthin: Sources, extraction, stability, biological activities and Its commercial applications - A review. Mar Drugs 12:128-152. doi: 10.3390/md12010128

Amundsen CR, Nordeide JT, Gjoen HM, B. L, Egeland ES (2015) Conspicuous carotenoid-based pelvic spine ornament in three-spined stickleback populations - occurrence and inheritance. Peerj 4:1-23. doi: 10.7717/peerj.872

Anbazahan SM et al. (2014) Immune response and disease resistance of carotenoids supplementation diet in *Cyprinus carpio* against *Aeromonas hydrophila*. Fish Shellfish Immunol 40:9-13. doi: 10.1016/j.fsi.2014.06.011

Aykanat T, Heath JW, Dixon B, Heath DD (2012) Additive, non-additive and maternal effects of cytokine transcription in response to immunostimulation with *Vibrio* vaccine in Chinook salmon (*Oncorhynchus tshawytscha*). Immunogenetics 64:691-703. doi: 10.1007/s00251-012-0624-2

Backström T, Brannas E, Nilsson J, Magnhagen C (2014) Behaviour, physiology and carotenoid pigmentation in Arctic charr *Salvelinus alpinus*. J. Fish Biol. 84:1-9. doi: 10.1111/jfb.12240

Backström T, Heynen M, Brannas E, Nilsson J, Magnhagen C (2015) Dominance and stress signalling of carotenoid pigmentation in Arctic charr (*Salvelinus alpinus*): Lateralization effects? Physiol Behav 138:52-57. doi: 10.1016/j.physbeh.2014.10.003

Balshine S (2012) Patterns of parental care in vertebrates. Evolution of Parental Care. Oxford University Press, New York

Barry TP, Malison JA, Held JA, Parrish JJ (1995) Ontogeny of the cortisol stress response in larval rainbow trout. Gen Comp Endocr 97:57-65. doi: 10.1006/Gcen.1995.1006

Bates D, Sarkar D (2007) lme4: Linear mixed-effects models using S4 classes R package version 0.99875-6

Bjerkeng B, Storebakken T, Liaaenjensen S (1992) Pigmentation of rainbow trout from start feeding to sexual maturation. Aquaculture 108:333-34
Black CA, Scott RJ, Bernards MA (2014) Seasonal changes in carotenoid and lipid storage by threespine stickleback, *Gasterosteus aculeatus*. Environmental Biology of Fishes 97:209-214. doi: 10.1007/s10641-013-0130-x
Blomhoff R, Blomhoff HK (2006) Overview of retinoid metabolism and function. J Neurobiol 66:606-630. doi: 10.1002/Neu.20242
Blount JD, Houston DC, Møller AP (2000) Why egg yolk is yellow. Trends in Ecology & Evolution 15:47-49. doi: 10.1016/S0169-5347(99)01774-7
Brazzola G, Chèvre N, Wedekind C (2014) Additive genetic variation for tolerance to estrogen pollution in natural populations of Alpine whitefish (*Coregonus* sp., Salmonidae). Evol Appl 7:1084–1093. doi: 10.1111/eva.12216
Brown AC, Cahn MD, Choi S, Clotfelter ED (2016) Dietary carotenoids and bacterial infection in wild and domestic convict cichlids (*Amatitlania* spp.). Environ Biol Fish 99:439-449. doi: 10.1007/s10641-016-0485-x
Candolin U, Tukiainen L (2015) The sexual selection paradigm: have we overlooked other mechanisms in the evolution of male ornaments? Proc R Soc Lond B Biol Sci 282:1-9. doi: 10.1098/rspb.2015.1987
Christiansen R, Torrissen OJ (1997) Effects of dietary astaxanthin supplementation on fertilization and egg survival in Atlantic salmon (*Salmo salar* L.). Aquaculture 153:51-62. doi: 10.1016/S0044-8486(97)00016-1
Clark ES, Wilkins LGE, Wedekind C (2013) MHC class I expression dependent on bacterial infection and parental factors in whitefish embryos (Salmonidae). Mol Ecol 22:5256-5269. doi: 10.1111/mec.12457
Clark ES, Pompini M, Marques da Cunha L, Wedekind C (2014) Maternal and paternal contributions to pathogen resistance dependent on development stage in a whitefish. Funct Ecol 28:714-723. doi: 10.1111/1365-2435.12214
Dale J, Dey CJ, Delhey K, Kempenaers B, Valcu M (2015) The effects of life history and sexual selection on male and female plumage colouration. Nature 527:367-370. doi: 10.1038/nature15509
Donnelly WA, Dill LM (1984) Evidence for crypsis in coho calmon, *Oncorhynchus kisutch* (Walbaum), parr - substrate color preference and chromatic reflectance. J Fish Biol 25:183-195. doi: 10.1111/J.1095-8649.1984.Tb04865.X
Einum S, Fleming IA (1999) Maternal effects of egg size in brown trout (*Salmo trutta*): norms of reaction to environmental quality. Proc R Soc Lond B Biol Sci 266:2095-2100. doi: 10.1098/rspb.1999.0893
Einum, S, Fleming, IA (2000) Highly fecund mothers sacrifice offspring survival to maximize fitness. Nature 405: 565-567. doi: 10.1038/35014600
Foote CJ, Brown GS, Hawryshyn CW (2004) Female colour and male choice in sockeye salmon: implications for the phenotypic convergence of anadromous and nonanadromous morphs. Anim Behav 67:69-83. doi: 10.1016/j.anbehav.2003.02.004
Garner SR, Neff BD, Bernards MA (2010) Dietary carotenoid levels affect carotenoid and retinoid allocation in female Chinook salmon *Oncorhynchus tshawytscha*. J Fish Biol 76:1474-1490. doi: 10.1111/J.1095-8649.2010.02579.X
Goodwin TW (1984) The biochemistry of carotenoids, II. Animals. Chapman & Hall, London

Grether GF (2000) Carotenoid limitation and mate preference evolution: A test of the indicator hypothesis in guppies (*Poecilia reticulata*). Evolution 54:1712-1724. doi: 10.1111/j.0014-3820.2000.tb00715.x

Ho ALFC, O'Shea SK, Pomeroy HF (2013) Dietary esterified astaxanthin effects on color, carotenoid concentrations, and composition of clown anemonefish, *Amphiprion ocellaris*, skin. Aquaculture International 21: 361-374. doi: 10.1007/s10499-012-9558-9

Jacob A, Evanno G, von Siebenthal BA, Grossen C, Wedekind C (2010) Effects of different mating scenarios on embryo viability in brown trout. Mol Ecol 19:5296-5307. doi: 10.1111/j.1365-294X.2010.04884.x

Janhunen M, Peuhkuri N, Primmer CR, Kolari I, Piironen J (2011) Does breeding ornamentation signal genetic quality in Arctic charr, *Salvelinus alpinus*? Evol Biol 38:68-78. doi: 10.1007/s11692-010-9100-9

Jensen LF, Hansen MM, Pertoldi C, Holdensgaard G, Mensberg KLD, Loeschcke V (2008) Local adaptation in brown trout early life-history traits: implications for climate change adaptability. Proc R Soc Lond B Biol Sci 275:2859-2868. doi: 10.1098/rspb.2008.0870

Johnstone RA (1997) The evolution of animal signals, fourth edn. Wiley-Blackwell, Hoboken, New Jersey

Kim S, Ha TY, Hwang IK (2009) Analysis, bioavailability, and potential healthy effects of capsanthin, natural red pigment from *Capsicum* spp. Food Rev Int 25:198-213. doi: 10.1080/87559120902956141

Kim S-Y, Velando A (2016) Genetic conflict between sexual signalling and juvenile survival in the three-spined stickleback. BMC Evol Biol 16:1-7. doi: 10.1186/s12862-016-0613-4

Kolluru GR et al. (2006) The effects of carotenoid and food availability on resistance to a naturally occurring parasite (*Gyrodactylus turnbulli*) in guppies (*Poecilia reticulata*). Biol J Linn Soc 89:301-309. doi: 10.1111/J.1095-8312.2006.00675.X

Kraaijeveld K, Kraaijeveld-Smit FJL, Komdeur J (2007) The evolution of mutual ornamentation. Anim Behav 74:657-677. doi: 10.1016/j.anbehav.2006.12.027

Krinsky NI, Yeum KJ (2003) Carotenoid-radical interactions. Biochem Bioph Res Co 305:754-760. doi: 10.1016/S0006-291x(03)00816-7

Lozano GA (2001) Carotenoids, parasites and sexual selection. Oikos 70:309-311. doi: 10.2307/3545643

Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer Associates Inc., Sunderland, Massachusetts

Marshall DJ, Bonduriansky R, Bussière LF (2008) Offspring size variation within broods as a bet-hedging strategy in unpredictable environments. Ecology 89:2506-2517. doi: 10.1890/07-0267.1

Nagler JJ, Parsons JE, Cloud JG (2000) Single pair mating indicates maternal effects on embryo survival in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 184:177-183. doi: 10.1016/S0044-8486(99)00309-9

Nordeide JT, Kekalainen J, Janhunen M, Kortet R (2013) Female ornaments revisited - are they correlated with offspring quality? J Anim Ecol 82:26-38. doi: 10.1111/1365-2656.12021

Nwachukwu ID, Udenigwe CC, Aluko RE (2016) Lutein and zeaxanthin: Production technology, bioavailability, mechanisms of action, visual function, and health claim status. Trends Food Sci Technol 49:74-84. doi: 10.1016/j.tifs.2015.12.005

OECD (1992) Guideline for the testing of chemicals 203 (fish, acute toxicity test), p 9
Olson VA, Owens IPF (1998) Costly sexual signals: are carotenoids rare, risky or required? Trends Ecol Evol 13:510-514. doi: 10.1016/s0169-5347(98)01484-0
Pailan GH, Sardar P, Mahapatra BK (2015) Marigold Petal Meal: A natural carotenoid source for pigmentation in Swordtail (*Xiphophorus helleri*). Anim Nutrition Feed Technol 15: 417-425. doi: 10.5958/0974-181X.2015.00042.6
Palace VP, Werner J (2006) Vitamins A and E in the maternal diet influence egg quality and early life stage development in fish: a review. Sci Mar 70:41-57.
Peters A (2007) Testosterone and carotenoids: an integrated view of trade-offs between immunity and sexual signalling. Bioessays 29:427-430. doi: 10.1002/Bies.20563
Pham MA, Byun HG, Kim KD, Lee SM (2014) Effects of dietary carotenoid source and level on growth, skin pigmentation, antioxidant activity and chemical composition of juvenile olive flounder *Paralichthys olivaceus*. Aquaculture 431: 65-72. doi: http://dx.doi.org/10.1016/j.aquaculture.2014.04.019
R Development Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria
Rice WR, Gaines SD (1994) Heads I win, tails you lose - testing directional alternative hypotheses in ecological and evolutionary research. Trends Ecol Evol 9:235-237. doi: 10.1016/0169-5347(94)90258-5
Rivera SM, Christou P, Canela-Garayoa R (2014) Identification of carotenoids using mass spectrometry. Mass Spectrom Rev 33:353-372. doi: 10.1002/mas.21390
Schiedt K, Vecchi M, Glinz E (1986) Astaxanthin and its metabolites in wild rainbow trout (*Salmo gairdneri* R.). Comp Biochem Phys B 83:9-12. doi: 10.1016/0305-0491(86)90324-X
Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671-675. doi: 10.1038/nmeth.2089
Seabra LMJ, Pedrosa LFC (2010) Astaxanthin: structural and functional aspects. Rev Nutr 23:1041-1050. doi: 10.1590/S1415-52732010000600010
Sheldon BC (2000) Differential allocation: Tests, mechanisms and implications. Trends Ecol Evol 15:397-402. doi: 10.1016/S0169-5347(00)01953-4
Sorensen AE, Mellor D, Jordan RC (2016) Effect of carotenoids on cichlid phenotype and mating behavior. Ethol Ecol Evol 28:77-84. doi: 10.1080/03949370.2015.1018953
Stelkens RB, Jaffuel G, Escher M, Wedekind C (2012) Genetic and phenotypic population divergence on a microgeographic scale in brown trout. Mol Ecol 21:2896-2915. doi: 10.1111/j.1365-294X.2012.05581.x
Stephensen CB (2001) Vitamin A, infection, and immune function. Annu Rev Nutr 21:167-192. doi: 10.1146/Annurev.Nutr.21.1.167
Steven DM (1949) Studies on animal carotenoids. 2. Carotenoids in the reproductive cycle of the brown trout. J Exp Biol 26:295-303.
Svensson PA, Pelabon C, Blount JD, Surai PF, Amundsen T (2006) Does female nuptial coloration reflect egg carotenoids and clutch quality in the Two-Spotted Goby (*Gobiusculus flavescens,* Gobiidae). Funct Ecol 20:689-698. doi: 10.1111/j.1365-2435.2006.01151.x
Svensson PA, Blount JD, Forsgren E, Amundsen T (2009) Female ornamentation and egg carotenoids of six sympatric gobies. J Fish Biol 75:2777-2787. doi: 10.1111/j.1095-8649.2009.02478.x

Thomas DB, McGraw KJ, Butler MW, Carrano MT, Madden O, James HF (2014) Ancient origins and multiple appearances of carotenoid-pigmented feathers in birds. Proc R Soc Lond B Biol Sci 281:1-9. doi: 10.1098/rspb.2014.0806

Tyndale ST, Letcher RJ, Heath JW, Heath DD (2008) Why are salmon eggs red? Egg carotenoids and early life survival of Chinook salmon (*Oncorhynchus tshawytscha*). Evol Ecol Res 10:1187-1199.

van den Berg H et al. (2000) The potential for the improvement of carotenoid levels in foods and the likely systemic effects. J Sci Food Agr 80:880-912. doi: 10.1002/(SICI)1097-0010(20000515)80:7<880::AID-JSFA646>3.0.CO;2-1

von Siebenthal BA, Jacob A, Wedekind C (2009) Tolerance of whitefish embryos to *Pseudomonas fluorescens* linked to genetic and maternal effects, and reduced by previous exposure. Fish Shellfish Immunol 26:531–535. doi: 10.1016/j.fsi.2009.02.008

Wedekind C, Meyer P, Frischknecht M, Niggli UA, Pfander H (1998) Different carotenoids and potential information content of red coloration of male three-spined stickleback. J Chem Ecol 24:787-801. doi: 10.1023/A:1022365315836

Wedekind C, Walker M, Portmann J, Cenni B, Müller R, Binz T (2004) MHC-linked susceptibility to a bacterial infection, but no MHC- linked cryptic female choice in whitefish. J Evol Biol 17:11-18. doi: 10.1046/j.1420-9101.2004.00669.x

Wedekind C, Jacob A, Evanno G, Nusslé S, Müller R (2008) Viability of brown trout embryos positively linked to melanin-based but negatively to carotenoid-based colours of their fathers. Proc R Soc Lond B Biol Sci 275:1737-1744. doi: 10.1098/rspb.2008.0072

Westley PAH, Stanley R, Fleming IA (2013) Experimental tests for heritable morphological color plasticity in non-native brown trout (*Salmo trutta*) populations. PLoS ONE 8. doi: 10.1371/journal.pone.0080401

Wilkins LGE, Rogivue A, Schütz F, Fumagalli L, Wedekind C (2015) Increased diversity of egg-associated bacteria on brown trout (*Salmo trutta*) at elevated temperatures. Sci Rep 5:1-15. doi: 10.1038/srep17084

Wilkins LGE, Fumagalli L, Wedekind C (2016) Effects of host genetics and environment on egg-associated microbiotas in brown trout (*Salmo trutta*). Mol Ecol 25:4930-4945. doi: 10.1111/mec.13798

Wilkins LGE, Marques da Cunha L, Glauser G, Vallat A, Wedekind C (2017) Environmental stress linked to consumption of maternally derived carotenoids in brown trout embryos (*Salmo trutta*). Ecol Evol. 7:1-12. doi: 10.1002/ece3.3076

Yi XW et al. (2015) Effects of dietary xanthophylls/astaxanthin ratios on the growth and skin pigmentation of large yellow croaker *Larimichthys crocea* (Richardson, 1846). J Appl Ichthyol 31:780-786. doi: 10.1111/jai.12763

Yuan JP, Peng JA, Yin K, Wang JH (2011) Potential health-promoting effects of astaxanthin: A high-value carotenoid mostly from microalgae. Mol Nutr Food Res 55:150-165. doi: 10.1002/mnfr.201000414

Tables

Table 1: The effects of treatment, egg carotenoid content, egg weight, and dam identity on embryo survival tested with likelihood ratio tests on mixed models logistic regressions.

Model terms	**Effect tested**	**AIC**	**d.f.**	χ^2	***P***
t + a + l + e + d		335	7		
t + a + t x a + l + e + d	t x a	335	8	1.2	0.27
t + a + l + t x l + e + d	t x l	336	8	0.6	0.44
t + a + l + e + t x e + d	t x e	337	8	0.03	0.86
t + a + l + e	d	355	6	22.5	**<0.001**
t + l + e + d	a	333	6	0.2	0.66
t + a + e + d	l	333	6	0.4	0.51
t + a + l + d	e	338	6	5.6	0.02
a + l + e + d	t	345	6	12.5	**<0.001**

Fixed effects: t, treatment; a, astaxanthin content (nM/egg); l, lutein content (nM/egg); e, egg weight. Random effect: d, dam. *P*-values < 0.05 and the reference model are highlighted in bold.

Table 2: The effects of treatment, egg carotenoid content, egg weight, and dam identity on (A) hatching time, (B) hatchling length and (C) yolk sac volume at hatching tested with likelihood ratio tests on mixed models regressions.

Model terms	**Effect tested**	**AIC**	**d.f.**	χ^2	***P***
(A) Hatching time					
t + a + l + e + d		8908	8		
t + a + t x a + l + e + d	t x a	8891	9	19.3	**<0.001**
t + a + l + t x l + e + d	t x l	8910	9	0.02	0.89
t + a + l + e + t x e + d	t x e	8897	9	13.3	**<0.001**
t + a + l + e	d	9974	7	1067	**<0.001**
t + l + e + d	a	8907	7	0.5	0.47
t + a + e + d	l	8907	7	0.4	0.54
t + a + l + d	e	8911	7	4.2	**0.04**
a + l + e + d	t	8961	7	54.2	**<0.001**
(B) Hatchling length					
t + a + l + e + d		1520	8		
t + a + t x a + l + e + d	t x a	1522	9	0.06	0.80
t + a + l + t x l + e + d	t x l	1522	9	0.02	0.88
t + a + l + e + t x e + d	t x e	1519	9	2.1	0.15
t + a + l + e	d	2152	7	634.3	**<0.001**
t + l + e + d	a	1518	7	0.3	0.55
t + a + e + d	l	1519	7	0.9	0.32
t + a + l + d	e	1520	7	1.7	0.19
a + l + e + d	t	1559	7	41.2	**<0.001**
(C) Yolk sac volume at hatching					
t + a + l + e + d		15816	8		
t + a + t x a + l + e + d	t x a	15818	9	0.02	0.89
t + a + l + t x l + e + d	t x l	15818	9	0.02	0.88
t + a + l + e + t x e + d	t x e	15816	9	2.5	0.11
t + a + l + e	d	17699	7	1885	**<0.001**
t + l + e + d	a	15814	7	0.05	0.82
t + a + e + d	l	15814	7	0.4	0.51
t + a + l + d	e	15818	7	4.5	**0.03**
a + l + e + d	t	15815	7	0.8	0.36

Fixed effects: t, treatment; a, astaxanthin content (nM/egg); l, lutein content (nM/egg); e, egg weight. Random effect: d, dam. *P*-values < 0.05 and reference models are highlighted in bold.

Table 3: The effects of treatment, egg carotenoid content, egg weight, and dam identity on larval growth tested with likelihood ratio tests on mixed models regressions.

Model terms	**Effect tested**	**AIC**	**d.f.**	χ^2	***P***
t + a + l + e + d		2793	8		
t + a + t x a + l + e + d	t x a	2792	9	3.2	0.07
t + a + l + t x l + e + d	t x l	2795	9	0.2	0.65
t + a + l + e + t x e + d	t x e	2786	9	8.3	**0.004**
t + a + l + e	d	2909	7	118.4	**<0.001**
t + l + e + d	a	2798	7	6.7	**0.009**
t + a + e + d	l	2792	7	1.6	0.21
t + a + l + d	e	2791	7	0.3	0.56
a + l + e + d	t	2796	7	5.5	0.02

Fixed effects: t, treatment; a, astaxanthin content (nM/egg); l, lutein content (nM/egg); e, egg weight. Random effect: d, dam. *P*-values < 0.05 and the reference model are highlighted in bold.

Figures

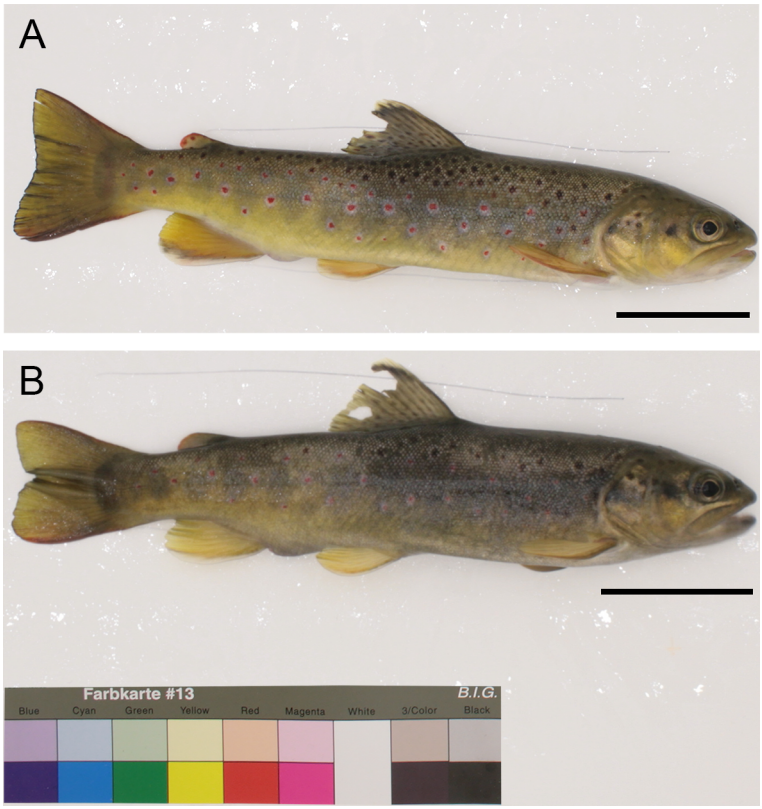

Fig. 1. Variation in female red colouration
Size-standardized examples of a female with (A) high and (B) low area of red spots relative to body area. The colour scale was pasted on panel B. Black lines represent 5 cm

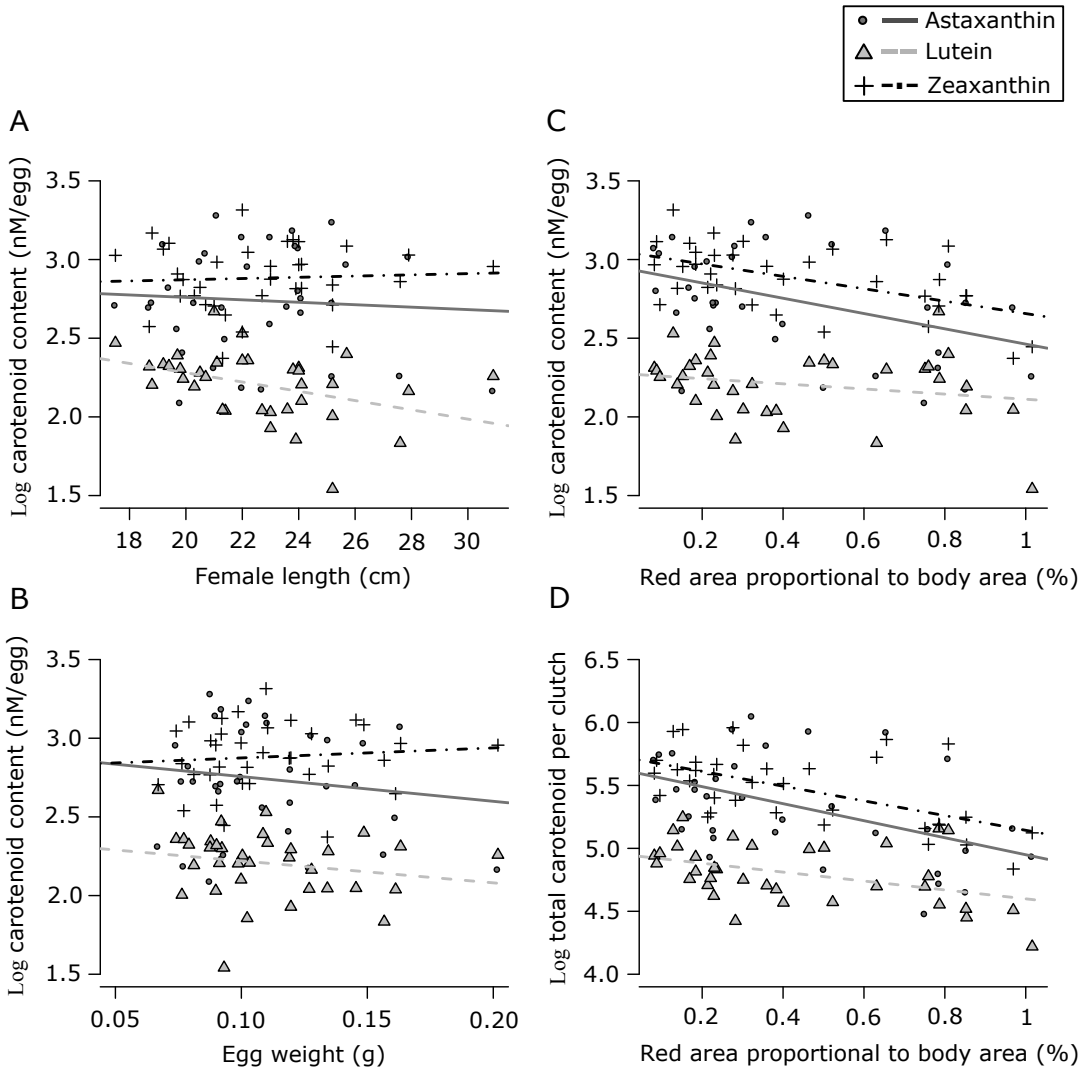

Fig. 2. Relationship between female (N=35) traits and egg carotenoid content
(A) Female length, (B) egg weight and (C) read area proportional to body area. In (D) female red area proportional to body area is compared to the total amount of carotenoid per clutch of eggs (*i.e.,* egg carotenoid content per egg is multiplied by the number of eggs). Carotenoid contents were log-transformed to show all three carotenoids in the same display. See results for statistics

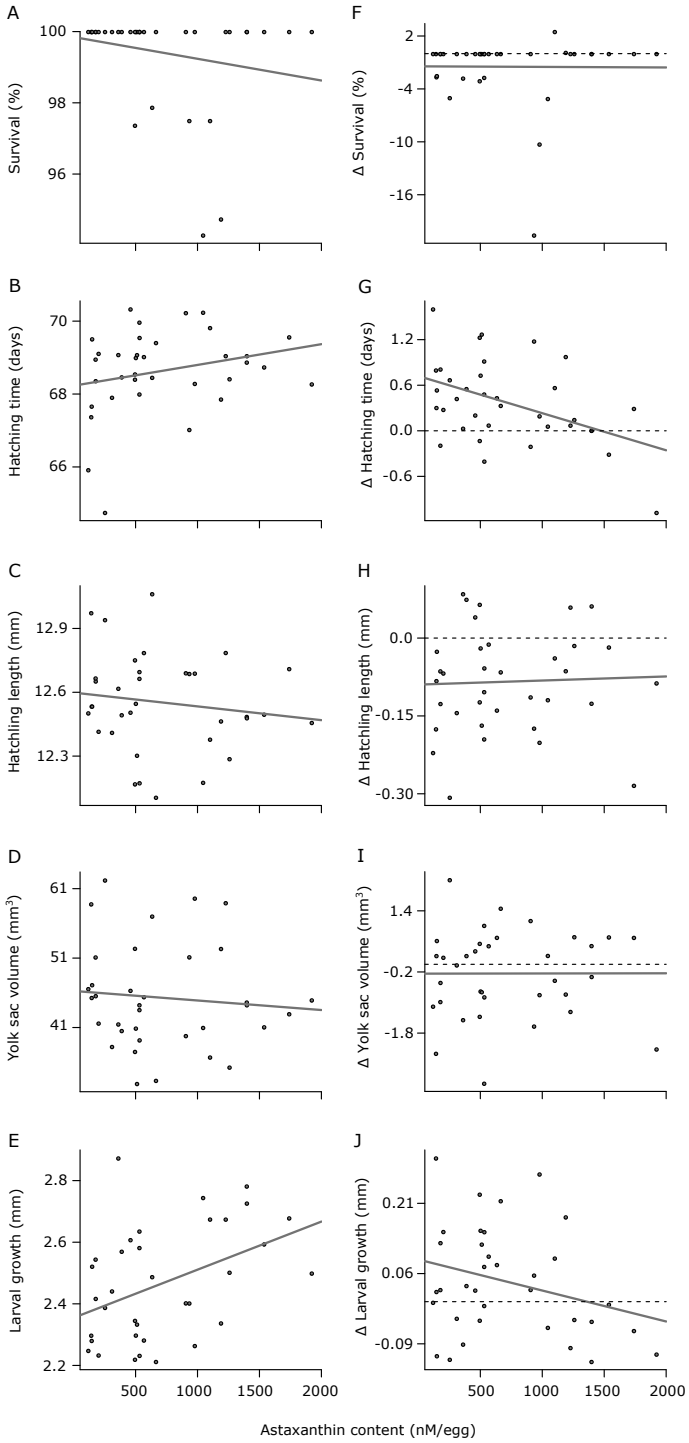

Fig. 3. Egg astaxanthin content and embryos' early fitness-related traits
(A) Embryo survival, (B) hatching time, (C) hatchling length, (D) yolk sac volume at hatching and (E) larval growth; A – E represent within the control treatment. The female-wise mean trait difference between *Pseudomonas fluorescens* treatment and control and its relationship with egg astaxanthin content are shown in the analogous panels F – J. Dashed lines represent when this difference is 0. In all panels, points represent female means (N=35) based on sibgroup means and solid lines give the regressions. See results for statistics

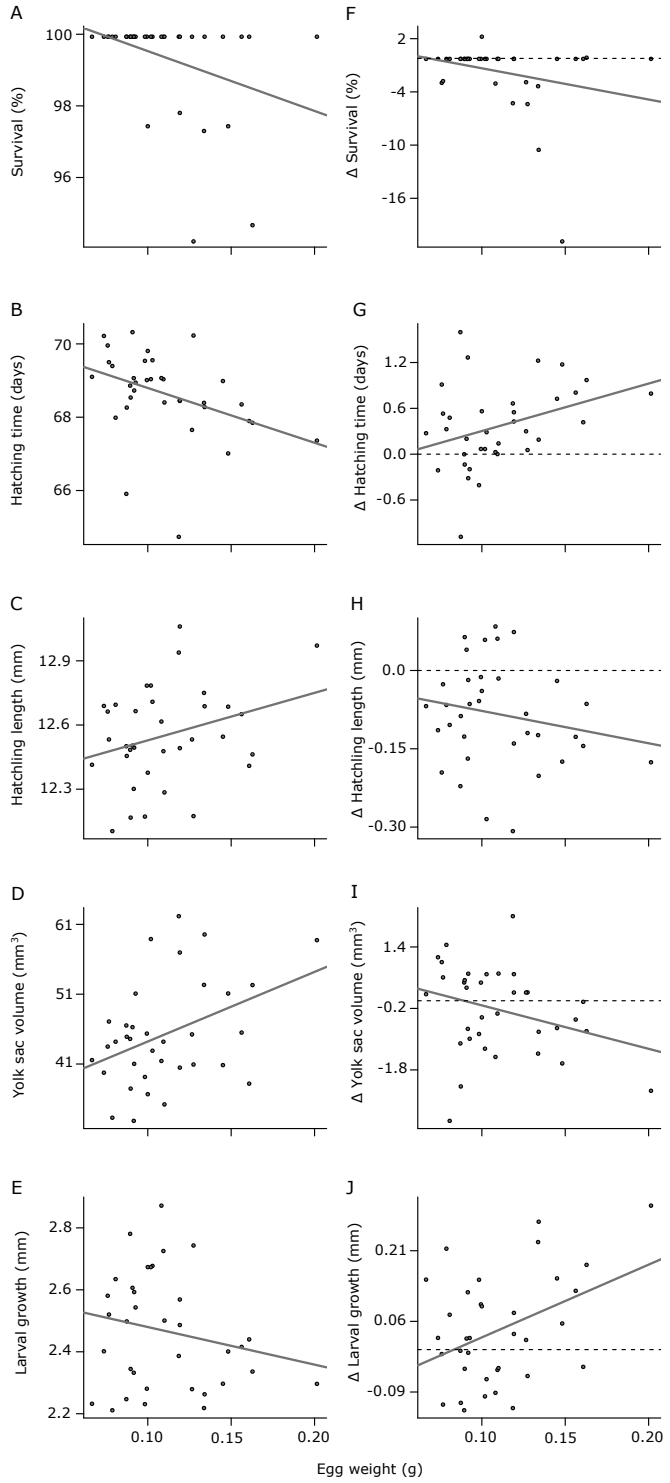

Fig. 4. Egg weight and embryos' early fitness-related traits
(A) Embryo survival, (B) hatching time, (C) hatchling length, (D) yolk sac volume at hatching and (E) larval growth; A – E represent within the control treatment. The female-wise mean trait difference between *Pseudomonas fluorescens* treatment and control and its relationship with egg weight are shown in the analogous panels F – J. Dashed lines represent when this difference is 0. In all panels, points represent female means (N=35) based on sibgroup means and solid lines give the regressions. See results for statistics

Supporting Material

Maternal allocation of carotenoids increases tolerance to bacterial infection in brown trout

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Table S1: Summary of mean concentrations (nM) of the four carotenoids measured in four unfertilized trout egg samples per female with associated standard deviations (SD) and coefficients of variation (CV). ND: Not detected.

	Lutein			Astaxanthin			Zeaxanthin			Capsanthin		
Sample	*Mean (nM)*	*SD (nM)*	*CV (%)*	*Mean (nM)*	*SD (nM)*	*CV (%)*	*Mean (nM)*	*SD (nM)*	*CV (%)*	*Mean (nM)*	*SD (nM)*	*CV (%)*
ACJ	820	34	4	4781	32	1	3706	96	3	3412	546	16
ACK	1003	143	14	3758	444	12	4869	514	11	10723	677	6
ACL	446	91	20	2032	416	20	5220	308	6	199	7	3
ACM	583	125	22	4203	610	15	4271	30	1	3838	584	15
ACN	646	220	34	6983	14	0	2060	53	3	ND	-	-
ACO	726	34	5	591	41	7	3614	302	8	4930	530	11
ACP	698	259	37	1036	19	2	2984	101	3	253	49	20
ACR	884	75	9	7713	1472	19	3851	5	0	276	41	15
ACS	799	52	7	6175	592	10	5348	19	0	11433	273	2
ACT	787	227	29	2559	611	24	5198	1232	24	4778	1358	28
ACU	404	50	12	2147	162	8	2757	509	18	3831	4	0
ACV	273	95	35	736	110	15	2896	419	14	132	11	8
ACW	429	52	12	5613	221	4	3623	24	1	2386	73	3
ACX	764	94	12	3935	623	16	2661	179	7	7867	1508	19
ACY	505	118	23	2292	389	17	3732	915	25	281	22	8
ACZ	1355	204	15	5617	1437	26	8263	1506	18	17872	1970	11
ADA	287	65	23	4934	8	0	2612	323	12	231	32	14
ADB	915	60	7	3647	185	5	4446	586	13	5255	108	2
ADC	139	-	-	732	-	-	1116	-	-	ND	-	-
ADD	437	53	12	1262	316	25	1777	219	12	ND	-	-
ADE	643	256	40	1859	80	4	2624	47	2	ND	-	-
ADF	339	148	44	1575	135	9	3001	257	9	280	59	21
ADG	717	86	12	4427	44	1	2067	265	13	3586	221	6
ADH	623	16	3	2147	73	3	2353	333	14	4441	7	0
AEK	807	134	17	496	7	1	2341	225	10	1147	293	26
AEL	1182	172	15	2069	221	11	4252	262	6	ND	-	
AEM	441	117	27	605	101	17	2357	186	8	554	127	23
AEN	913	196	21	620	43	7	1385	107	8	1359	338	25
AEP	1872	83	4	828	95	11	2027	90	4	2055	155	8
AEQ	983	102	10	1466	264	18	3238	204	6	3137	670	21
AER	639	150	24	2152	118	5	5897	760	13	ND	-	-
AES	835	166	20	2004	325	16	1500	66	4	1864	185	10
AET	864	155	18	5057	235	5	4656	210	5	183	1	0
AEU	842	139	17	2681	167	6	5075	755	15	1806	389	22
AEV	444	94	21	2003	343	17	942	403	43	ND	-	-

Table S2: The effects of treatment, egg carotenoid concentrations, egg weight, and dam identity on embryo survival tested by likelihood ratio tests on mixed models logistic regressions.

Model terms	**Effect tested**	**AIC**	**d.f.**	χ^2	***P***
t + a + l + e + d		335	7		
t + a + t x a + l + e + d	t x a	336	8	1.2	0.28
t + a + l + t x l + e + d	t x l	337	8	0.5	0.49
t + a + l + e + t x e + d	t x e	337	8	0.03	0.85
t + a + l + e	d	363	6	29.9	**<0.001**
t + l + e + d	a	335	6	0.06	0.81
t + a + e + d	l	333	6	0	0.99
t + a + l + d	e	335	6	3	0.08
a + l + e + d	t	346	6	12.4	**<0.001**

Fixed effects: t, treatment; a, astaxanthin concentration (nM/g); l, lutein concentration (nM/g); e, egg weight. Random effect: d, dam. *P*-values < 0.05 and the reference model are highlighted in bold.

Table S3: The effects of treatment, egg carotenoid concentrations, egg weight, and dam identity on (A) hatching time, (B) hatchling length and (C) yolk sac volume at hatching tested by likelihood ratio tests on mixed models regressions.

Model terms	**Effect tested**	**AIC**	**d.f.**	χ^2	***P***
(A) Hatching time					
t + a + l + e + d		8909	8		
t + a + t x a + l + e + d	t x a	8881	9	29.6	**<0.001**
t + a + l + t x l + e + d	t x l	8910	9	0.9	0.34
t + a + l + e + t x e + d	t x e	8898	9	13.3	**<0.001**
t + a + l + e	d	9984	7	1077.1	**<0.001**
t + l + e + d	a	8907	7	0.3	0.57
t + a + e + d	l	8907	7	0.1	0.80
t + a + l + d	e	8909	7	2.6	0.10
a + l + e + d	t	8961	7	54.2	**<0.001**
(B) Hatchling length					
t + a + l + e + d		1520	8		
t + a + t x a + l + e + d	t x a	1522	9	0.1	0.70
t + a + l + t x l + e + d	t x l	1522	9	0.3	0.57
t + a + l + e + t x e + d	t x e	1520	9	2.0	0.16
t + a + l + e	d	2155	7	637.1	**<0.001**
t + l + e + d	a	1518	7	0.3	0.58
t + a + e + d	l	1519	7	1.1	0.30
t + a + l + d	e	1519	7	0.4	0.52
a + l + e + d	t	1559	7	41.2	**<0.001**
(C) Yolk sac volume at hatching					
t + a + l + e + d		15816	8		
t + a + t x a + l + e + d	t x a	15818	9	0.2	0.65
t + a + l + t x l + e + d	t x l	15817	9	0.5	0.47
t + a + l + e + t x e + d	t x e	15815	9	2.6	0.11
t + a + l + e	d	17684	7	1871.5	**<0.001**
t + l + e + d	a	15814	7	0.2	0.63
t + a + e + d	l	15814	7	0.7	0.41
t + a + l + d	e	15816	7	1.9	0.16
a + l + e + d	t	15815	7	0.8	0.36

Fixed effects: t, treatment; a, astaxanthin concentration (nM/g); l, lutein concentration (nM/g); e, egg weight. Random effect: d, dam. *P*-values < 0.05 and reference models are highlighted in bold.

Table S4: The effects of treatment, egg carotenoid concentrations, egg weight, and dam identity on larval growth tested by likelihood ratio tests on mixed models regressions.

Model terms	**Effect tested**	**AIC**	**d.f.**	χ^2	***P***
t + a + l + e + d		2793	8		
t + a + t x a + l + e + d	t x a	2790	9	5.2	**0.02**
t + a + l + t x l + e + d	t x l	2795	9	0.05	0.83
t + a + l + e + t x e + d	t x e	2787	9	8.3	**0.004**
t + a + l + e	d	2908	7	117.5	**<0.001**
t + l + e + d	a	2798	7	6.76	**0.009**
t + a + e + d	l	2793	7	2.1	0.15
t + a + l + d	e	2791	7	0.3	0.58
a + l + e + d	t	2791	7	6.2	**0.01**

Fixed effects: t, treatment; a, astaxanthin concentration (nM/g); l, lutein concentration (nM/g); e, egg weight. Random effect: d, dam. *P*-values < 0.05 and the reference model are highlighted in bold.

Supporting Figures

Figure S1: Calibration curves of astaxanthin, capsanthin, lutein, and zeaxanthin in the 1-50 nM concentration range

The average peak area of three replicate injections for each concentration was used. Calibration curves were fitted with a polynomial order 2 equation with $R^2 > 0.996$ for all carotenoids.

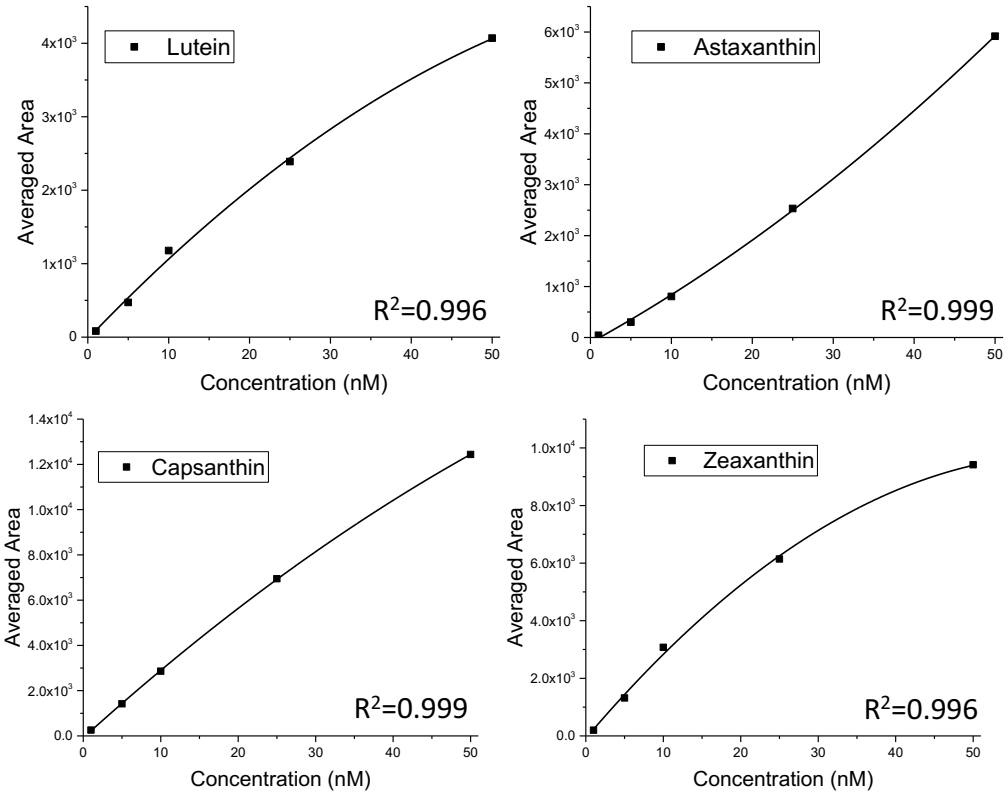
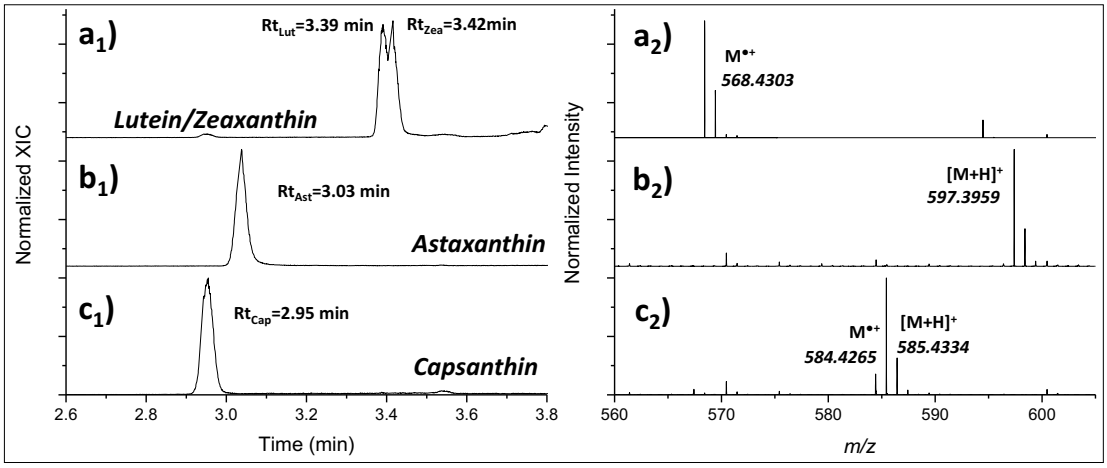


Figure S2: UHPLC-ESI-QTOF mass spectrometry
(a1), (b1) and (c1) correspond to Extracted Ion Chromatograms (XIC) of *m/z* 568.4275, *m/z* 597.3938 and *m/z* 585.4302 for lutein/zeaxanthin, astaxanthin and capsanthin respectively (MEW of ±25 ppm). (a2), (b2) and (c2) correspond to extended views in the *m/z* 560-610 mass range of mass spectra acquired at 3.40 min (lutein/zeaxanthin), 3.03 min (astaxanthin) and 2.95 min (capsanthin), respectively.



Carotenoid	MF	Rt (min)	Ion species	Theoretical ion	Measured ion	Mass error (ppm)	LLOQ (nM)
Capsanthin	$C_{40}H_{56}O_3$	2.95	$M^{+\cdot}$ / $[M+H]^+$	584.4224 / 585.4302	584.4265 / 585.4334	6.0 / 4.4	3.0
Astaxanthin	$C_{40}H_{52}O_4$	3.03	$[M+H]^+$	597.3938	597.3959	3.4	2.5
Lutein	$C_{40}H_{56}O_2$	3.39	$M^{+\cdot}$	568.4275	568.4303	4.9	1.0
Zeaxanthin	$C_{40}H_{56}O_2$	3.42	$M^{+\cdot}$	568.4275	568.4293	3.2	1.0

Figure S3: Relationship between female traits and egg capsanthin content
(A) Female length, (B) egg weight and (C) read area proportional to body area. In (D) female red area proportional to body area is compared to the total amount of carotenoid per clutch of eggs (*i.e.* egg carotenoid content per egg is multiplied by the number of eggs). Capsanthin content was log-transformed. See Results for statistics.

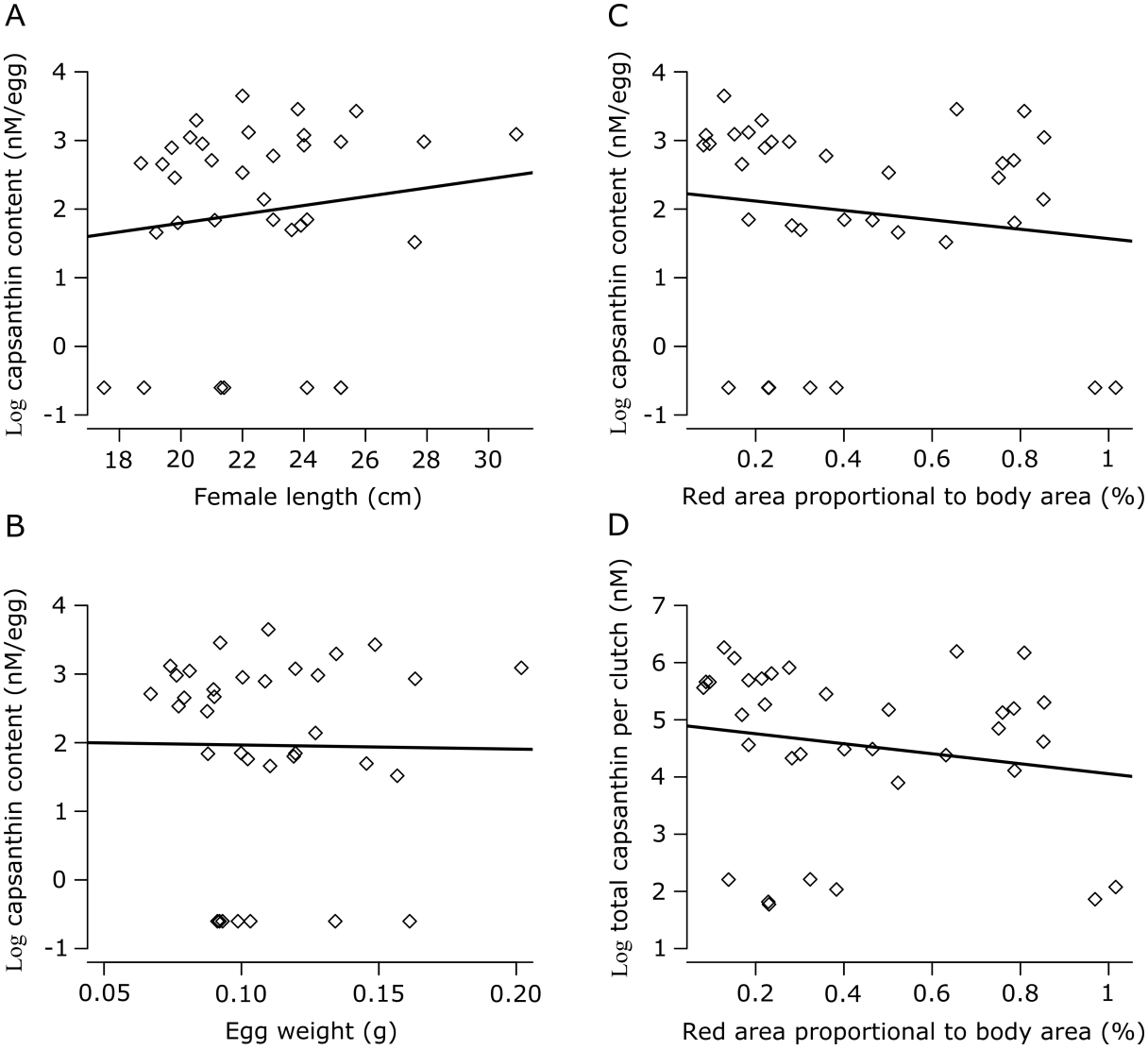


Figure S4: Relationship between egg redness and egg carotenoid content
Carotenoid contents were log-transformed to show all three carotenoids in the same display. Statistics: egg redness *vs*. astaxanthin (r = 0.54, $p < 0.001$); egg redness *vs*. lutein: (r = -0.09, $p = 0.6$) and egg redness *vs*. zeaxanthin (r = 0.17, $p = 0.34$).

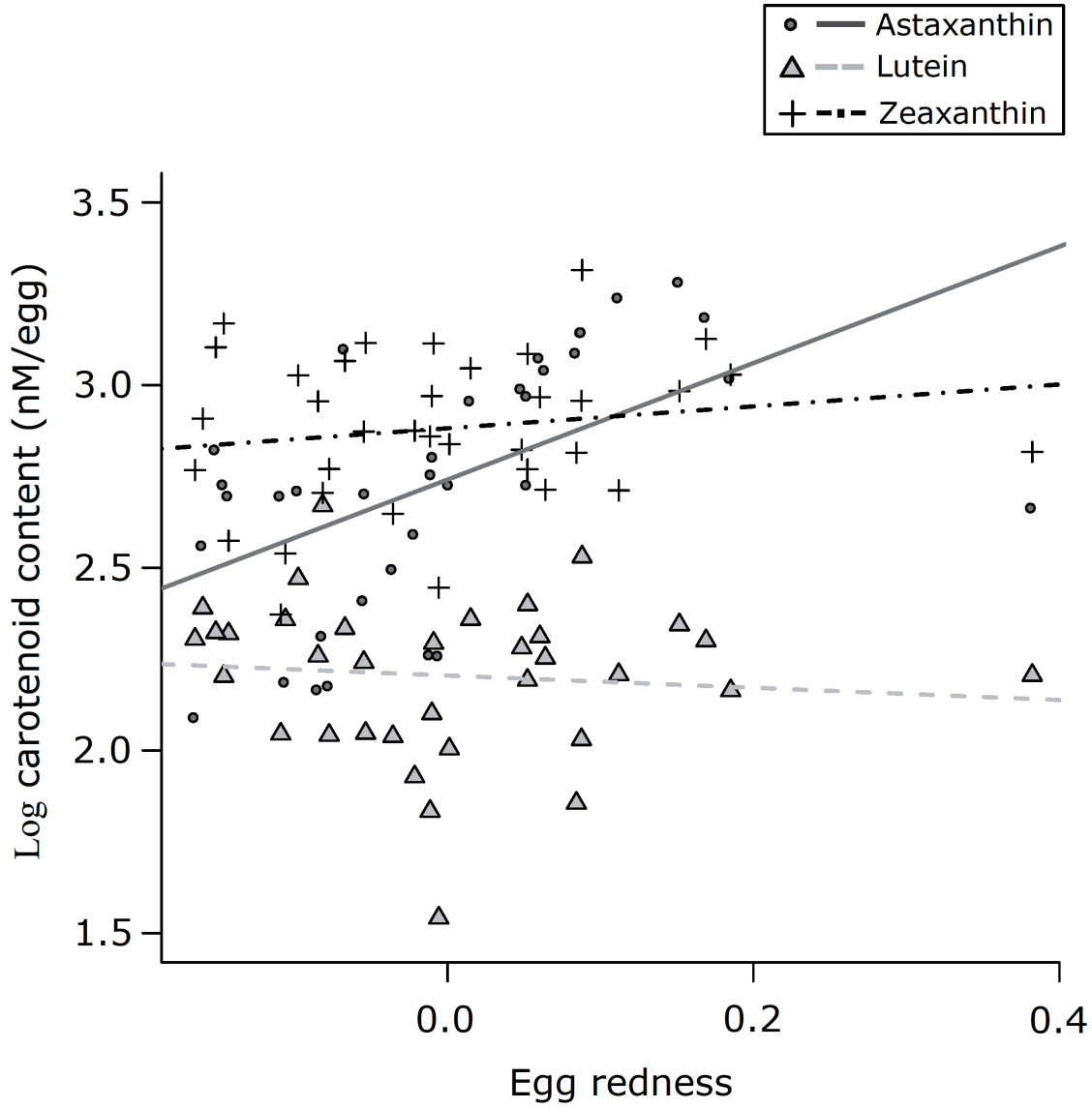


Figure S5: Relationship between egg redness and egg capsanthin content
Capsanthin content was log-transformed. Statistics: r = 0.14, p = 0.93.

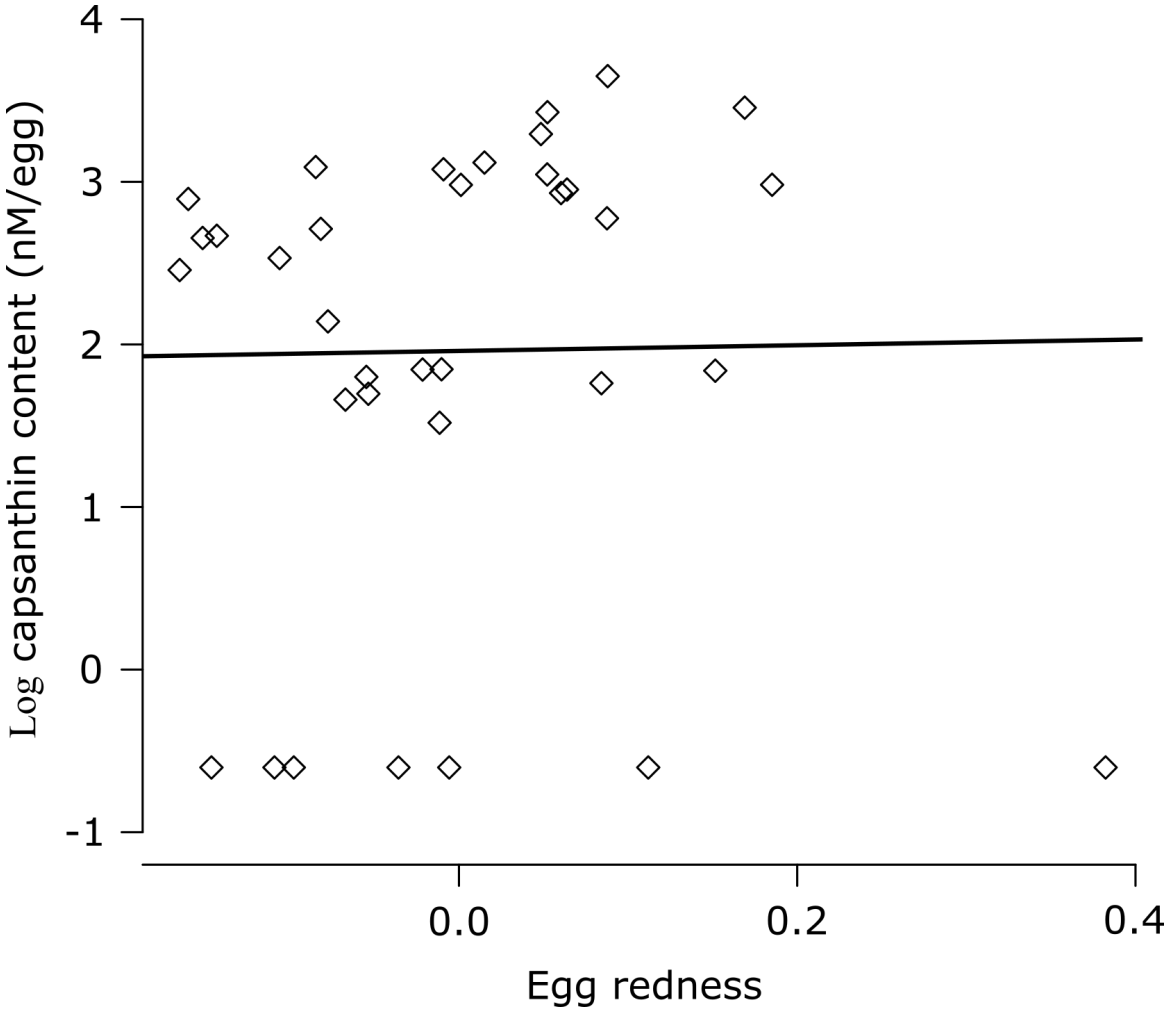


Figure S6: Relationship between female length and egg weight
Statistics: ($r = 0.51$, $p = 0.002$).

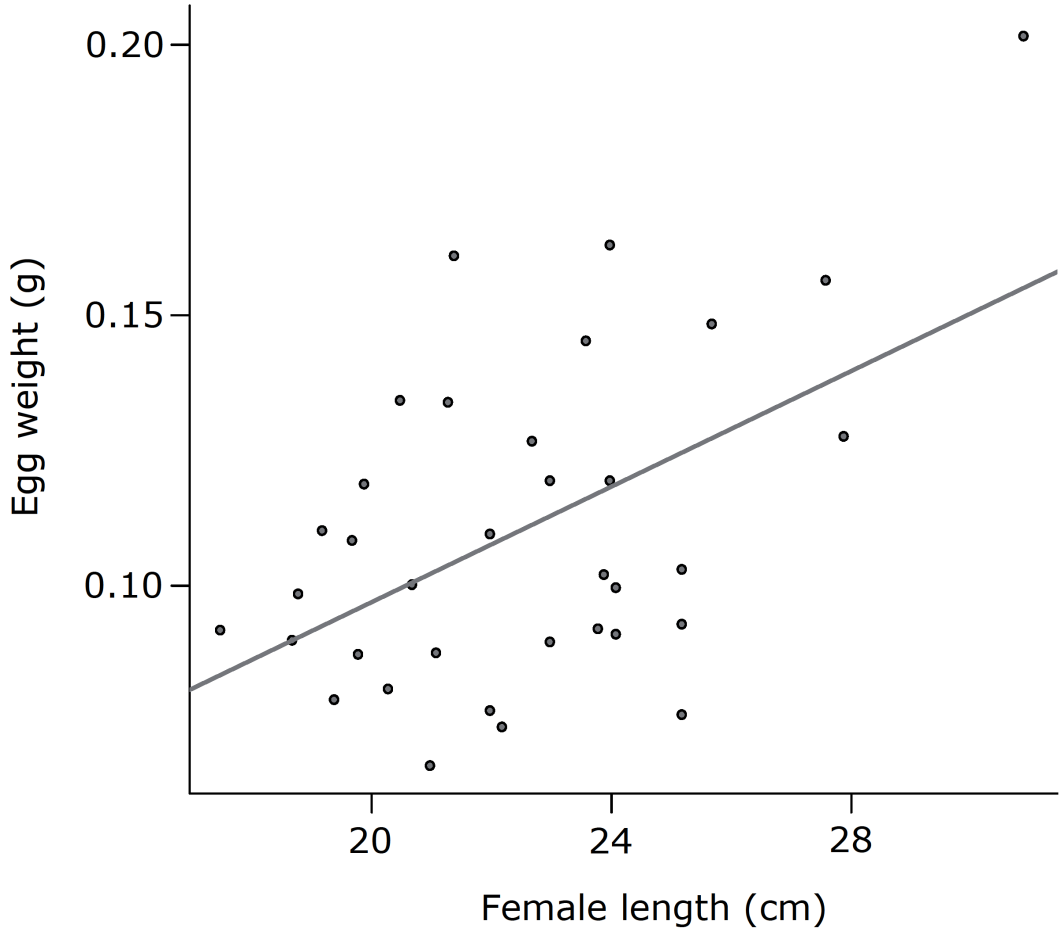
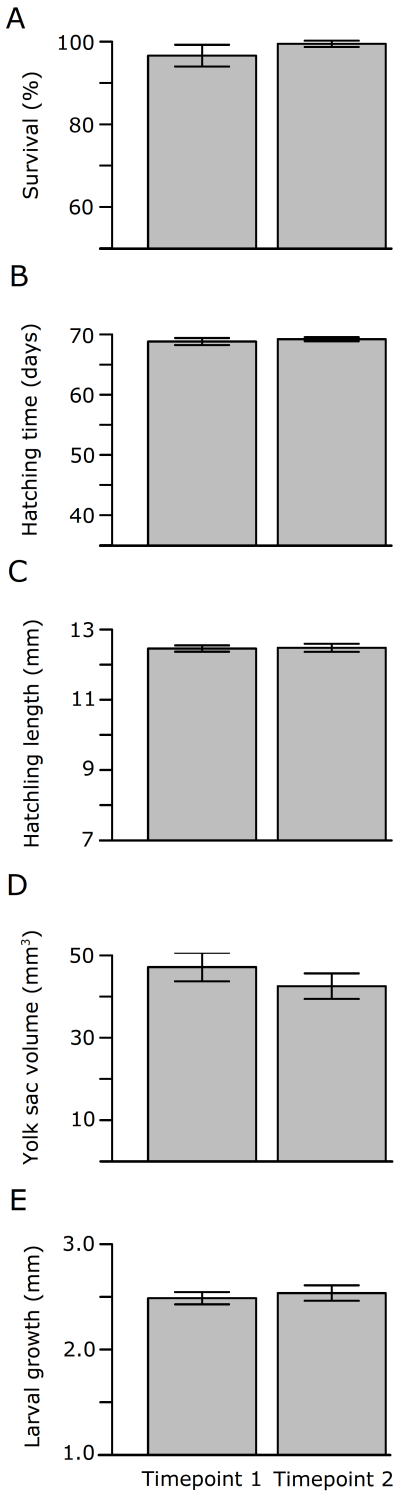


Figure 7: Effects of time point of infection with *Pseudomonas fluorescens* on embryo early fitness-related traits
(A) Embryo survival, (B) hatching time, (C) hatchling length, (D) yolk sac volume at hatching and (E) larval growth. Error bars are 95% confidence intervals. See Results for statistics.



Macro for color analyses

For the measures of carotenoid based red coloration (further referred to as red spots) of females, we designed a macro in ImageJ v.1.49u. First, the white balance of the image was readjusted based on the mean values of the black and the white area of the color scale in each of the three color channels (RGB; see Eq. 1). The image was then duplicated and split in three color channels in the RGB color space. The difference between the red and the green channels was computed with the Image Calculator function and the contrast of the resulting image was amplified by adjusting window and levels. The red spots were then separated by thresholding the resulting image and added to the ROI Manager with the Analyse Particles function. The area (in pixels), the median values and the mean values in each color channel were measured in the original image for each ROI (*i.e.*, each red spots). The RGB values obtained were transformed into the CIE-Lab color space in Microsoft Excel via a two-step transformation through the XYZ color space (RWG Hunt, 1991 ; León et al., 2006; see Eq. 2 and 3).

Equation 1: Adjustment of the white balance of the image in the RGB (0-255) color space

$$V' = (V_i - b_i)\left(\frac{255}{b_i - w_i}\right)$$

Where: V'= new value of the pixel ; V= original value of the pixel ; b= measured value of the black reference ; w=measured value of the white reference and i= the color channel (red, green and blue).

Equation 2: Transformation from the RGB (0-255) color space to the XYZ color space

a)

$$Var_{R,G,B} = \begin{pmatrix} R \\ G \\ B \end{pmatrix} \begin{cases} 0.4124\left(\dfrac{\frac{a}{255} + 0.055}{1.055}\right)^{2.4} & | \quad \text{if } \dfrac{a}{255} > 0.04045 \\ \dfrac{a}{255 \times 12.92} & | \quad \text{if } \dfrac{a}{255} \leq 0.04045 \end{cases}$$

b)

$$\begin{aligned} X &= 41.24 \times Var_R + 35.76 \times Var_G + 18.05 \times Var_B \\ Y &= 21.26 \times Var_R + 71.52 \times Var_G + 7.22 \times Var_B \\ Z &= 1.93 \times Var_R + 11.92 \times Var_G + 95.05 \times Var_B \end{aligned}$$

Where: Var is an intermediate variable derived from R, G and B respectively, by replacing a by R, G and B in the conditional equation a).

Equation 3: Transformation from the XYZ color space to the CIE-L*a*b* color space.

$$L^* = \begin{cases} 116\left(\dfrac{Y}{Y_{ref}}\right)^{1/3} - 16 & | \; if \; \dfrac{Y}{Y_{ref}} > 0.008856 \\ 903.3\left(\dfrac{Y}{Y_{ref}}\right) & | \; if \; \dfrac{Y}{Y_{ref}} \leq 0.008856 \end{cases}$$

$$a^* = 500\left[\left(\frac{X}{X_{ref}}\right)^{1/3} - \left(\frac{Y}{Y_{ref}}\right)^{1/3}\right]$$

$$b^* = 200\left[\left(\frac{Y}{Y_{ref}}\right)^{1/3} - \left(\frac{Z}{Z_{ref}}\right)^{1/3}\right]$$

Where: X_{ref} Y_{ref} and Z_{ref} correspond to the length of the axes in the XYZ color space, respectively 95.047, 100 and 108.883

References:

León, K., Mery, D., Pedreschi, F., León, J., 2006. Color measurement in L*a*b* units from RGB digital images. Food Research International 39, 1084–1091. doi:10.1016/j.foodres.2006.03.006

Hunt, R.W.G, 1987. Measuring color. Chichester, England: Ellis Horwood. 2nd ed. 1991