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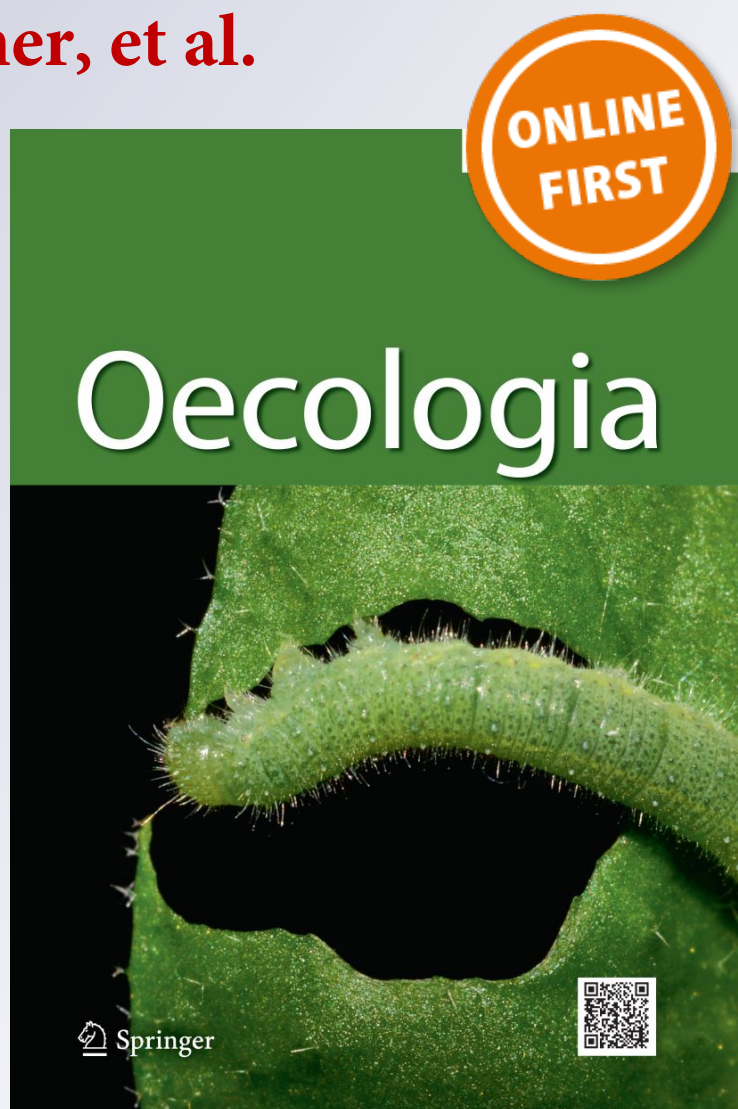
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# Maternal allocation of carotenoids increases tolerance to bacterial infection in brown trout

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**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 ( $n = 2960$ ), 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.

**Keywords** 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

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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: (1) antioxidant activity against damage caused by free radicals (Krinsky and Yeum 2003), (2) improved pathogen resistance by increasing the production of antibodies and the proliferation of immune cells (Blount et al. 2000; Peters 2007), and (3) 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 Torrisen 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 not only 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.

## Materials 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  $-80^{\circ}\text{C}$ . The remaining gametes were used for full-factorial in vitro fertilizations following the

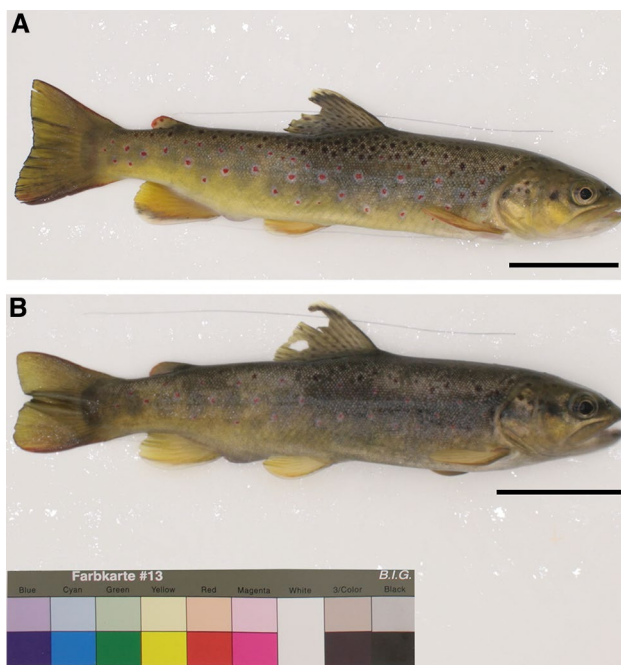
protocols described in Jacob et al. (2010). After 2 h of egg hardening, standardized photos were taken for egg counting and measurements of egg colours. We produced five different breeding blocks consisting of five females crossed with five males each for the river Kiese (i.e.  $5 \times 25$  families) and two blocks consisting of six females crossed with five males each for the river Rotache (i.e.  $2 \times 30$  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 (OECD 1992). Standard water had been autoclaved, tempered, and oxygenated before use. Embryos were raised at  $6.5^{\circ}\text{C}$  in a climate chamber with a 12-h 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  $a^*$



**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 (colour figure online)

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 × 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 programme (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 s/spectra. The TOF analyser was calibrated using a solution of sodium formate (0.01 mg/L in isopropanol/H<sub>2</sub>O 90:10). A mass accuracy better than 10 ppm was achieved using a leucine-enkephalin solution as lock-mass [200 pg/µL in ACN/H<sub>2</sub>O (50:50)] infused continuously using the LockSpray source. Source settings were as follows: cone, 25 V; 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 12 × 32 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  $m/z_{\text{theor}}$  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<sup>3</sup>) 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 (1) these carotenoids were significantly correlated to astaxanthin content, and (2) 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  $\leq 0.01$  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  $[M]^+$  (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

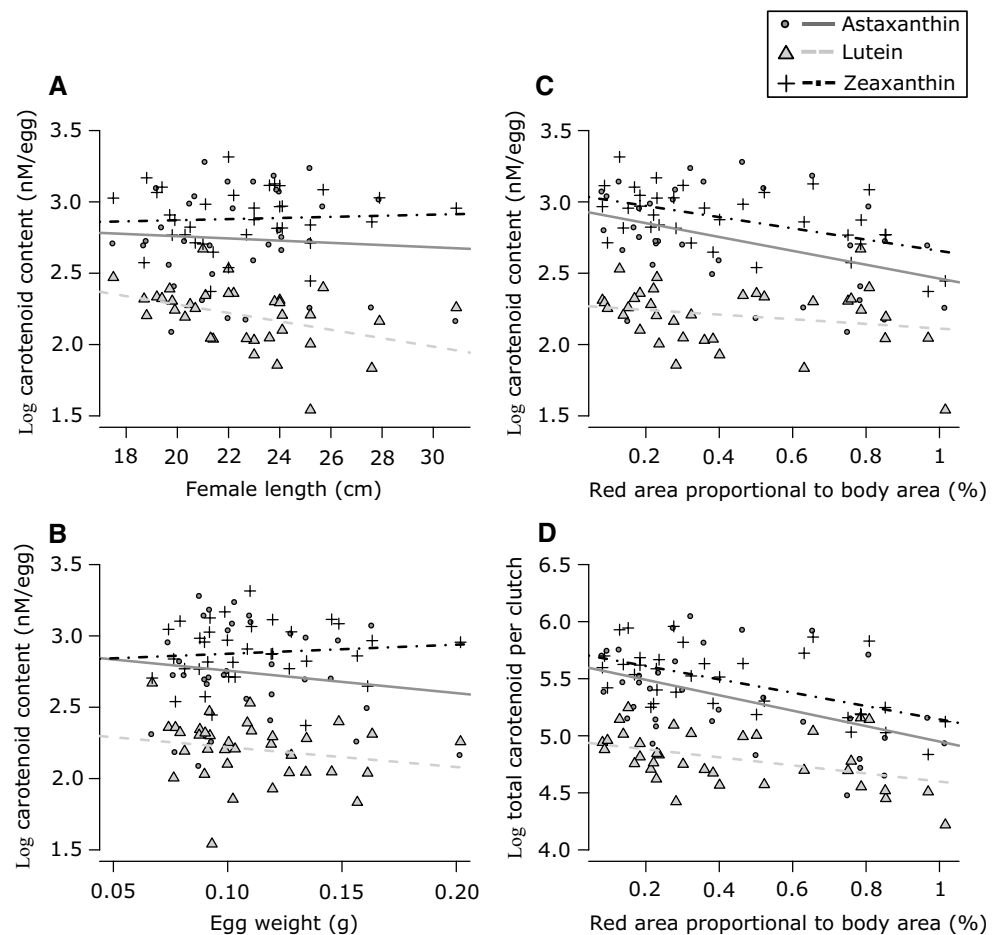
Astaxanthin, lutein and zeaxanthin were found in the eggs of all 35 females in the following amounts (means  $\pm$  95% confidence intervals): astaxanthin (720 nM/egg  $\pm$  165), lutein (178 nM/egg  $\pm$  27) and zeaxanthin (848 nM/egg  $\pm$  129). Capsanthin could only be detected above threshold level in 28 females and showed wide confidence intervals (700 nM/egg  $\pm$  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, S3 A–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 and 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 and 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



**Fig. 2** Relationship between female ( $N = 35$ ) traits and egg carotenoid content. **a** Female length, **b** egg weight and **c** red area proportional to body area. In **d** female red area proportional to body area is compared to the total amount of carotenoids 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 (colour figure online)



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 (Tables 1, 2, 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

**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	df	$\chi^2$	<i>P</i>
t + a + l + e + d		335	7		
t + a + t × a + l + e + d	t × a	335	8	1.2	0.27
t + a + l + t × l + e + d	t × l	336	8	0.6	0.44
t + a + l + e + t × e + d	t × e	337	8	0.03	0.86
t + a + l + e	d	355	6	22.5	<b>&lt;0.001</b>
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	<b>&lt;0.001</b>

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

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 (Tables 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, 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 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	df	$\chi^2$	<i>P</i>
(A) Hatching time					
t + a + l + e + d		8908	8		
t + a + t × a + l + e + d	t × a	8891	9	19.3	<b>&lt;0.001</b>
t + a + l + t × l + e + d	t × l	8910	9	0.02	0.89
t + a + l + e + t × e + d	t × e	8897	9	13.3	<b>&lt;0.001</b>
t + a + l + e	d	9974	7	1067	<b>&lt;0.001</b>
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	<b>0.04</b>
a + l + e + d	t	8961	7	54.2	<b>&lt;0.001</b>
(B) Hatchling length					
t + a + l + e + d		1520	8		
t + a + t × a + l + e + d	t × a	1522	9	0.06	0.80
t + a + l + t × l + e + d	t × l	1522	9	0.02	0.88
t + a + l + e + t × e + d	t × e	1519	9	2.1	0.15
t + a + l + e	d	2152	7	634.3	<b>&lt;0.001</b>
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	<b>&lt;0.001</b>
(C) Yolk sac volume at hatching					
t + a + l + e + d		15,816	8		
t + a + t × a + l + e + d	t × a	15,818	9	0.02	0.89
t + a + l + t × l + e + d	t × l	15,818	9	0.02	0.88
t + a + l + e + t × e + d	t × e	15,816	9	2.5	0.11
t + a + l + e	d	17,699	7	1885	<b>&lt;0.001</b>
t + l + e + d	a	15,814	7	0.05	0.82
t + a + e + d	l	15,814	7	0.4	0.51
t + a + l + d	e	15,818	7	4.5	<b>0.03</b>
a + l + e + d	t	15,815	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	df	$\chi^2$	P
t + a + l + e + d		2793	8		
t + a + t × a + l + e + d	t × a	2792	9	3.2	0.07
t + a + l + t × l + e + d	t × l	2795	9	0.2	0.65
t + a + l + e + t × e + d	t × e	2786	9	8.3	<b>0.004</b>
t + a + l + e	d	2909	7	118.4	<b>&lt;0.001</b>
t + l + e + d	a	2798	7	6.7	<b>0.009</b>
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

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, 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, h; Table 2B). Yolk sac volume was not significantly affected by either treatment or egg weight (Fig. 4d, 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, 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 centre of the astaxanthin molecule (Ho et al. 2013; 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).

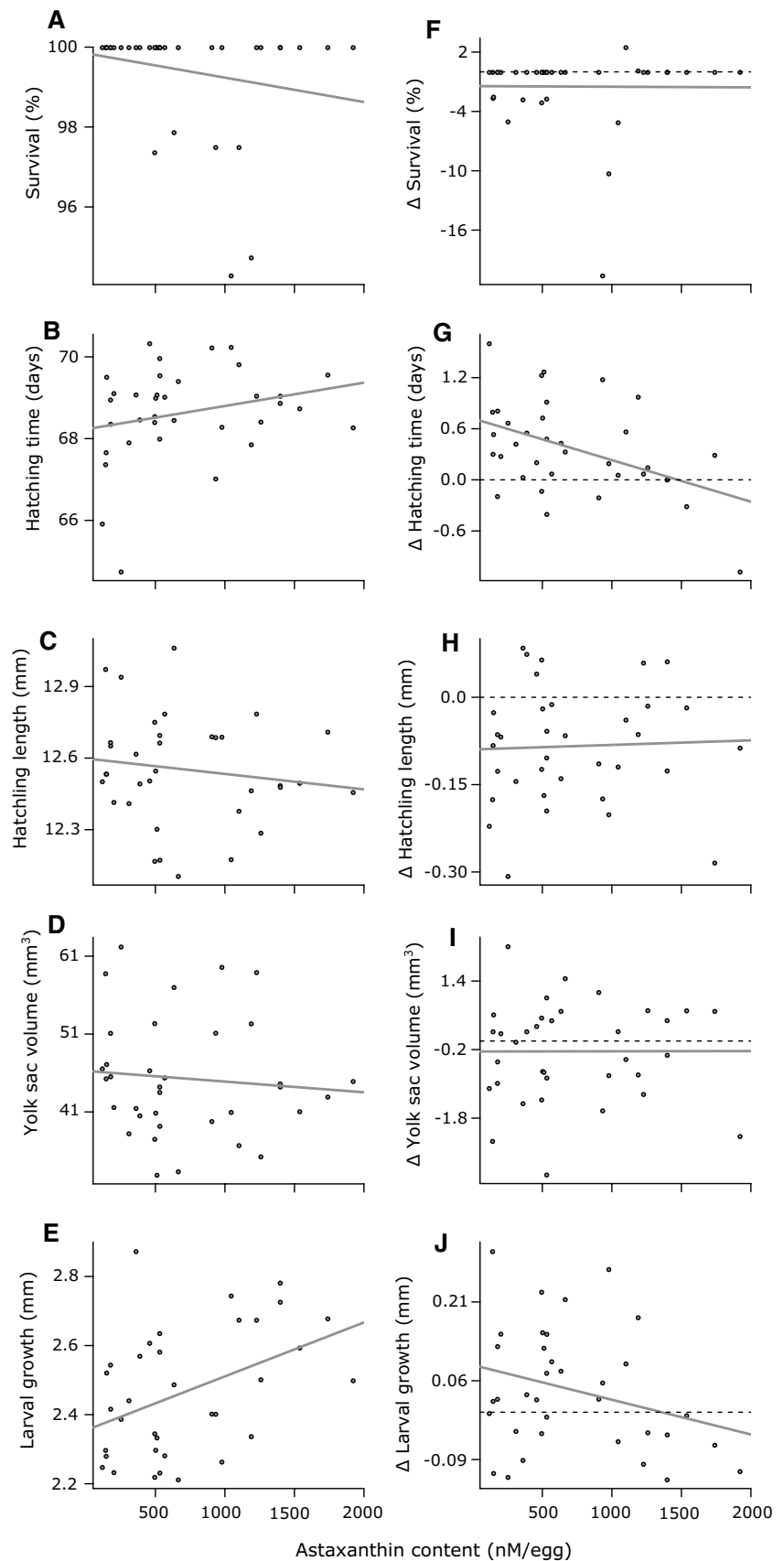
### Treatment effects

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, 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, 2014). The hatchlings of the exposed group were smaller but with similar yolk sac volumes.

### Carotenoid effects during pathogen stress

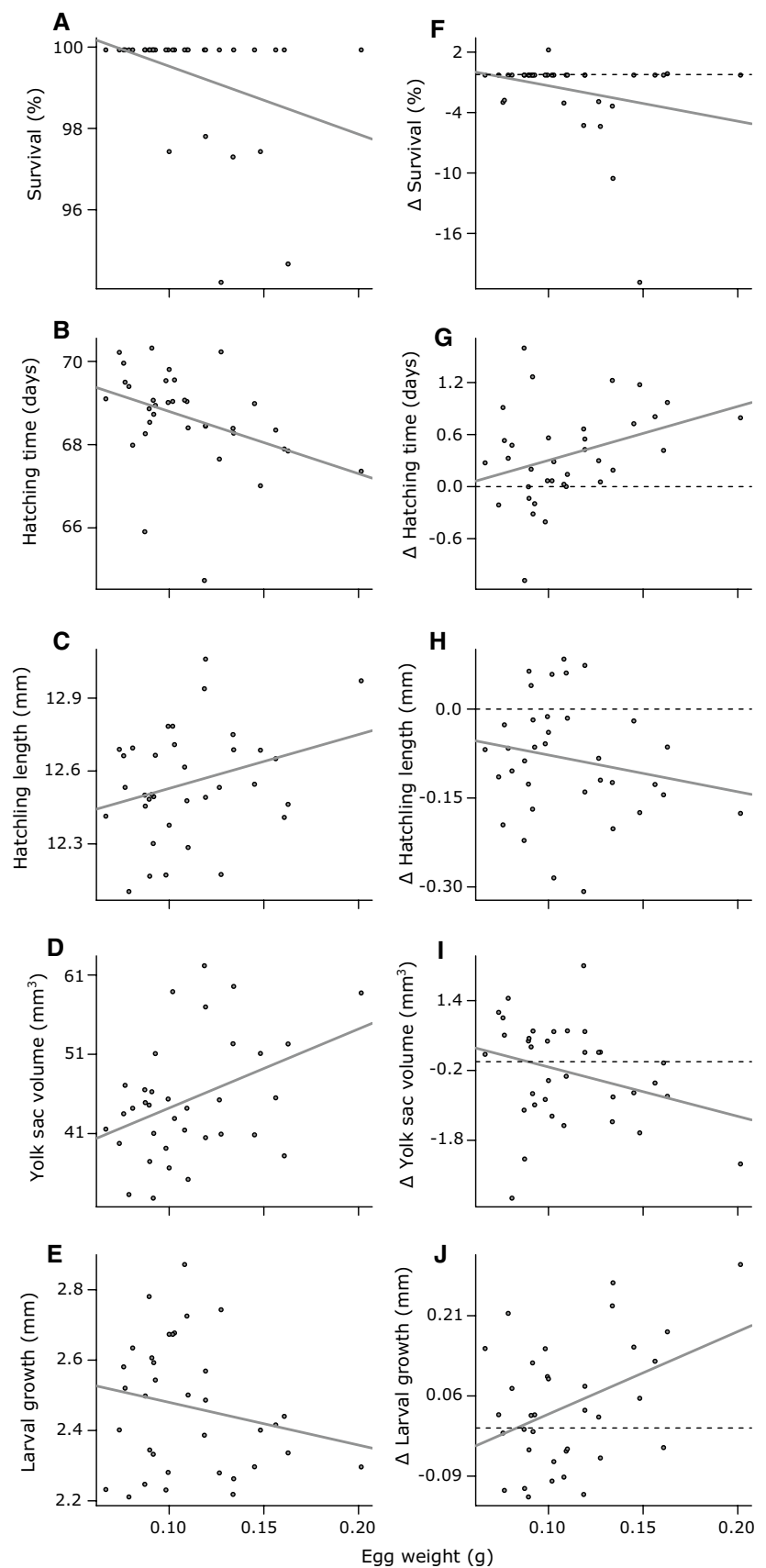
There were no correlations between lutein and embryo or larval development, neither under the stressed nor under the non-stressed condition. We only found a correlation between lutein content in the egg and female size, as well as female skin red colouration, that both remain to be further studied.

**Fig. 3** Egg astaxanthin content and embryos' early fitness-related traits. **a** Embryo survival, **b** hatching time, **c** hatching 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



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 centre 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 have also been found in various salmonids (Bjerkeng et al. 1992; Garner et al. 2010; Backström et al. 2014, 2015) who can show much phenotypic plasticity in their skin colourations (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](https://doi.org/10.5061/dryad.sj416).

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**Author contribution statement** 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.

## 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*. *Aquac Int* 23:1239–1248. doi:[10.1007/s10499-015-9880-0](https://doi.org/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](https://doi.org/10.3390/md12010128)
- Amundsen CR, Nordeide JT, Gjoen HM, Larsen B, 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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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 Endocrinol* 97:57–65. doi:[10.1006/Gcen.1995.1006](https://doi.org/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, Liaenjen Jensen S (1992) Pigmentation of rainbow trout from start feeding to sexual maturation. *Aquaculture* 108:333–334
- Black CA, Scott RJ, Bernards MA (2014) Seasonal changes in carotenoid and lipid storage by threespine stickleback, *Gasterosteus aculeatus*. *Environ Biol Fishes* 97:209–214. doi:[10.1007/s10641-013-0130-x](https://doi.org/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](https://doi.org/10.1002/Neu.20242)
- Blount JD, Houston DC, Møller AP (2000) Why egg yolk is yellow. *Trends Ecol Evol* 15:47–49. doi:[10.1016/S0169-5347\(99\)01774-7](https://doi.org/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](https://doi.org/10.1111/eva.12216)
- Brown AC, Leonard HM, McGraw KJ, Clotfelter ED (2014) Maternal effects of carotenoid supplementation in an ornamented cichlid fish (*Amantitlania siquia*). *Funct Ecol* 28:612–620. doi:[10.1111/1365-2435.12205](https://doi.org/10.1111/1365-2435.12205)
- Brown AC, Cahn MD, Choi S, Clotfelter ED (2016) Dietary carotenoids and bacterial infection in wild and domestic convict cichlids (*Amantitlania* spp.). *Environ Biol Fish* 99:439–449. doi:[10.1007/s10641-016-0485-x](https://doi.org/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](https://doi.org/10.1098/rspb.2015.1987)
- Christiansen R, Torrisen 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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/10.1038/nature15509)
- Donnelly WA, Dill LM (1984) Evidence for crypsis in coho salmon, *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](https://doi.org/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](https://doi.org/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](https://doi.org/10.1038/35014600)
- Footo 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](https://doi.org/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](https://doi.org/10.1111/J.1095-8649.2010.02579.X)
- Goodwin TW (1984) The biochemistry of carotenoids, II. Animals. Chapman and 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](https://doi.org/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. *Aquac Int* 21:361–374. doi:[10.1007/s10499-012-9558-9](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/10.1098/rspb.2008.0870)
- Johnstone RA (1997) The evolution of animal signals, 4th edn. Wiley-Blackwell, Hoboken
- 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](https://doi.org/10.1186/s12862-016-0613-4)
- 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](https://doi.org/10.1080/87559120902956141)
- 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](https://doi.org/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](https://doi.org/10.1016/j.anbehav.2006.12.027)

- Krinsky NI, Yeum KJ (2003) Carotenoid-radical interactions. *Biochem Biophys Res Co* 305:754–760. doi:[10.1016/S0006-291x\(03\)00816-7](https://doi.org/10.1016/S0006-291x(03)00816-7)
- Lozano GA (2001) Carotenoids, parasites and sexual selection. *Oikos* 70:309–311. doi:[10.2307/3545643](https://doi.org/10.2307/3545643)
- Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer Associates Inc., Sunderland
- 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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/10.1016/j.tifs.2015.12.005)
- OECD (1992) Guideline for the testing of chemicals 203 (fish, acute toxicity test), p 9. Organisation for Economic Cooperation and Development, Paris. <http://www.oecd.org>
- 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](https://doi.org/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](https://doi.org/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](https://doi.org/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:[10.1016/j.aquaculture.2014.04.019](https://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
- 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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/10.1038/nmeth.2089)
- Seabra LMJ, Pedrosa LFC (2010) Astaxanthin: structural and functional aspects. *Rev Nutr* 23:1041–1050. doi:[10.1590/S1415-52732010000600010](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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 Agric* 80:880–912. doi:[10.1002/\(SICI\)1097-0010\(20000515\)80:7<880::AID-JSFA646>3.0.CO;2-I](https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<880::AID-JSFA646>3.0.CO;2-I)
- 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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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*. doi:[10.1371/journal.pone.0080401](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://doi.org/10.1002/mnfr.201000414)