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Das Gelbe vom Ei: The evolutionary significance of carotenoid-mediated egg coloration in the three-spined stickleback

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Introduction

1. Basic study background and aims

Carotenoids have been shown to play an important role in sexual selection of the three-spined stickleback *Gasterosteus aculeatus*, as the males' red breeding coloration is based on these chemical compounds. Stickleback females lay clutches differing in their more or less yellowish egg coloration. The intensity of egg color is most likely a product of carotenoids as well, since the presence of egg carotenoids has been confirmed in some other fish species. Carotenoids are widely believed to be beneficial for the organism obtaining them, since they have antioxidant function and play a role in immuno-stimulation and regulation. In my study I investigated the influence of dietary carotenoid level on carotenoid investment of stickleback females into their eggs. Furthermore I looked for fitness differences in offspring in dependence of the carotenoid level their mothers had received. I tested whether the effect of carotenoids on offspring fitness might depend on rearing environment. Last, I aimed to find out if there is a link between the carotenoid investment a stickleback male is able to make into his red breeding coloration and his daughter's investment of carotenoids into her eggs.

2. Maternal effects

The genotype can be described as representing a "library" of hereditary information passed on from parent to offspring (Bonduriansky & Day, 2009). However, parents can influence their offspring's condition not only indirectly (genetically) but also directly (Qvarnstrom & Price, 2001). Nongenetic mechanisms of inheritance have been observed in all taxonomic groups for a large variety of phenotypic traits (reviewed in Youngson & Whitelaw, 2008). The vertical transmission of factors other than DNA (i.e. epigenetic, somatic, cytoplasmatic) can influence an offspring's phenotype on basis of its genotype (Bonduriansky & Day, 2009). Maternal effects can be described as developmental influences across life cycle stages leading to phenotypic differences between offspring as a result of genetic or environmental differences in the maternal generation (Mousseau & Dingle, 1991). Maternal effects can be

determined at different times in the development of the offspring: before ovulation through the cytoplasm of the egg, during the gestation period after fertilisation through the reproductive tract, or after birth/hatching through various types of maternal care (McLaren, 1981). In several species, maternal effects seem to be especially important in early life stages of the offspring but loosing importance later in life (Bernardo, 1996a). For example, in the life-bearing fish *Poecilia parae* maternal effects are a major determinant of early offspring body size and thus time of sexual maturation, but have no influence on late life-history traits e.g. adult longevity (Lindholm *et al.*, 2006). However, it is typically the early life-history traits such as growth rate and survival which receive highest attention when studying maternal effects. Summarizing, females have the opportunity to influence life-history traits of their offspring by factors other than DNA, like for example egg characteristics (e.g. egg size, egg contents).

3. Egg characteristics in oviparous animals

The importance of egg quality as a maternal effect is especially obvious in oviparous animals where females invest all the resources necessary for embryonic development into the egg (Blount *et al.*, 2002). The influence of egg size on offspring fitness has been one of the most intensely studied maternal effects in the past decades (Bernardo, 1996b). In bird species, evidence for a positive influence of egg size on traits such as hatchability, growth and offspring survival and a correlation of egg size with maternal condition has been found in some studies but not in others (reviewed by Williams, 1994). Also in fish some examples of a positive influence of egg size on offspring viability are known. In the brown trout *Salmo trutta* juveniles hatched from large eggs had growth and survival advantages over siblings from small eggs under semi-natural conditions (Einum & Fleming, 1999). In the Persian sturgeon *Acipenser persicus* a correlation was found between egg size and larval weight and volume of the yolk sac (Nazari *et al.*, 2009). Recently the influence of specific egg components independent of egg size on offspring fitness has received more and more attention. Canary (*Serinus canaria*) hens incorporate testosterone into their eggs and this hormone is linked to aggressive behavior and social status in the

juvenile offspring (Schwabl, 1993). In lesser black-backed gulls *Larus fuscus*, artificially extending clutch size beyond the usual number of three eggs did not lead to a reduction of egg size in the surplus eggs. However, the surplus eggs contained relatively less lipid which had a negative effect on the chicks' probability of survival until fledging, thus making the importance of lipid incorporation into the egg visible (Nager *et al.*, 2000). In coho salmon *Onchorhynchus kisutch* and ambon damselfish *Pomacentrus amboinensis* cortisol levels of freshly ovulated eggs reflect the circulating cortisol levels in the adult female (Stratholt *et al.*, 1997; McCormick, 1998). Maternal cortisol administration has been shown to affect the offspring's behavioral responses in a novel environment in the atlantic salmon *Salmo salar* (Espmark *et al.*, 2008) and under predator exposure in the three-spined stickleback (Giesing *et al.*, 2011). Thus, certain components mothers add to their eggs can have a big impact on the offspring's viability, especially in early life stages.

4. The functions of carotenoids and the importance of carotenoid investment as a maternal effect

Many egg-laying animals incorporate carotenoids into their eggs. The influence of carotenoids on offspring life-history traits has been investigated mainly in birds (e.g. Blount *et al.*, 2002; Bortolotti *et al.*, 2003; Royle *et al.*, 2003; Biard *et al.*, 2007; Berthouly *et al.*, 2008; De Neve *et al.*, 2008; Ewen *et al.*, 2009; Morales *et al.*, 2011) but also on some fish species (e.g. Christiansen & Torrissen, 1997; Tachibana *et al.*, 1997; Verakunpiriya *et al.*, 1997; Amar *et al.*, 2004; Ahmadi *et al.*, 2006; Grether *et al.*, 2008; Garner *et al.*, 2010; Lakeh *et al.*, 2010). Carotenoids, divided according to their molecular structure into xanthophylls and carotenes, are a group of over 600 biochemicals which can be synthesized by bacteria, algae, fungi and plants, but must be obtained through the diet by animals (summarized in Møller *et al.*, 2000). Carotenoids have no nutritional value apart from their potential to be vitamin A precursors (Bendich, 1993). They are believed to play an important role in immuno-regulation and immuno-stimulation in vertebrates: carotenoids have been shown to enhance T and B lymphocyte proliferation, increase number and enhance tumor cell killing ability of cytotoxic T cells and macrophages, increase the number of T-helper

cells, increase the production of tumor necrosis factor and stimulate the production of various cytokines and interleukins in mammals (Bendich, 1989; Bendich & Olson, 1989; Bendich, 1993; Chew, 1993; Møller *et al.*, 2000). Some carotenoids upregulate the *connexin43* gene expression which increases intercellular gap junctional communication (Burri, 2000). Furthermore, carotenoids have antioxidant function: they act as quenchers of singlet molecular oxygen and free radical traps. Singlet oxygen is generated by electronic energy transfer and its presence can lead to DNA damage or lipid peroxidation in biological systems (Edge *et al.*, 1997). By quenching electron exchange energy transfer, which is the prime carotenoid photoprotection mechanism and leads to deactivation of singlet oxygen, a carotenoid triplet state is produced which can easily return to its ground state by converting the energy into heat or being quenched itself (Stahl & Sies, 1993; Edge *et al.*, 1997). Free radicals - atoms or molecules with unpaired electrons - are produced during immune response as an effective weapon against bacterial and other invaders. However, in the course of this protective mechanism killing the pathogens, free radicals can be overproduced (oxidative stress) and may cause damage to the immune system and other cells (Bendich, 1989). Possible consequences of oxidative stress are destruction of cellular membranes (change of membrane properties like fluidity and flexibility as well as membrane functions like intracellular signalling), cellular proteins and nucleic acids, which can result in immune system incompetence (Chew, 1996; Chew & Park, 2004). Thus, by quenching free radicals, carotenoids protect lipids from oxidation, decrease immuno-supressive peroxides and maintain membrane receptors necessary for proper immune function (Møller *et al.*, 2000). Finally, retinoids, metabolic derivates of carotenoids, are important for tissue repair, morphogenesis and pattern formation as well as in regulation of immune response genes in vertebrates. Carotenoid based coloration could advertise retinoid resources (Hartley & Kennedy, 2004; Costantini & Moller, 2008).

Embryos are rapid growing organisms with high levels of oxidative metabolism, potentially leading to oxidative stress, thus making the maternal investment of carotenoids into the egg especially important in early life stages of the offspring (Møller *et al.*, 2000). Carotenoid concentration in embryonic tissue has been shown to reach the maximum level in late embryonic development and

at hatching in birds (Surai *et al.*, 1996). The carotenoid content of the yolk sac membrane decreases significantly by over 50% during the hatching period (which is considered as oxidative stress) and liver carotenoids are rapidly depleted in the neonatal period in different domestic poultry species (Surai *et al.*, 1996; Surai *et al.*, 1998). In fish and other aquatic egg laying animals, little is known about antioxidant or carotenoid use in early life stages. In the sea bass *Dicentrarchus labrax* high carotenoid concentrations (i.e. Vitamin E) were found in eggs, embryos and the early stage of larval development. Vitamin E decreased slowly but continuously during larval growth (Guerriero *et al.*, 2003). Summarizing, carotenoids have numerous functions which can be beneficial for the organism. The possession of carotenoid resources might be especially crucial in early life stages were oxidative stress is high.

5. The carotenoid trade-off hypothesis

The carotenoid trade-off hypothesis predicts that under conditions of carotenoid scarcity, individuals face a trade-off between physiological utilization of carotenoids and their deposition into ornaments (Lin *et al.*, 2010; Vinkler & Albrecht, 2010). Carotenoid scarcity might arise because of individual differences in foraging efficiency or in the level of parasitism and diseases which inhibit carotenoid absorption or demand carotenoid supply for antioxidant activity and immune function (Olson & Owens, 1998; Blount *et al.*, 2002). In species where males are brightly orange-red colored while females are dull, the strategies for antioxidant use might be different for the two sexes, with females facing the decision about how much carotenoids to invest into their eggs versus their immune system and antioxidant defence mechanisms (Møller *et al.*, 2000). In fact, the females' investment of carotenoids into eggs might lead to an additional trade-off, namely between ornament and eggs, resulting in females being more dull than males (Nordeide *et al.*, 2008). Feeding a carotenoid supplemented diet to the mother significantly increases the carotenoid concentrations in the female's plasma and certain tissues of the eggs in several species. In the lesser black-backed gull *Larus fuscus* yolk carotenoid levels were higher in eggs laid by females fed a carotenoid enriched diet versus eggs laid by control females. Furthermore, yolk carotenoid content declined over the egg

laying sequence independent of carotenoid level of the diet and the decline of carotenoid levels between the two last laid eggs was higher in dull females than in bright colored ones, making the explanation that carotenoid content of plumage and egg are associated likely (Blount *et al.*, 2002). In red-legged partridges *Alectoris rufa* the proportions of the major types of carotenoids in the mothers' diet were reflected in their eggs' yolk and a high carotenoid diet resulted in significantly higher carotenoid concentration in the egg in comparison with the low carotenoid diet (Bortolotti *et al.*, 2003). In the hihi *Notiomystis cincta* females fed on carotenoid enriched diets incorporated more carotenoids into egg yolk than control females and maternal deposition of carotenoids into the egg compensated the costs arising from experimentally induced parasitism, with fledglings of high-carotenoid diet mothers showing increased growth and ultimate nestling size (Ewen *et al.*, 2009). The trade-off between physiological use of carotenoids and their deposition into ornaments is not limited to birds, but also exist in other egg laying species, like fish. In rainbow trout *Onchorhynchus mykiss* the astaxanthin concentration in the maternal feed and in her eggs was significantly correlated. Higher dietary carotenoid levels had positive influence on fertilization rate, percentage of eyed eggs, percentage of hatched eggs and specific growth rate of the offspring (Ahmadi *et al.*, 2006; Lakeh *et al.*, 2010). Similar results of astaxanthin supplementation of females were found in Atlantic cod *Gadhus morhua* (Sawanboonchun *et al.*, 2008). Several other studies, however, find no positive relationship between maternal diet and egg carotenoid content and/or carotenoid-mediated effect on the tested fitness parameters in offspring (rainbow trout *Onchorhynchus mykiss*, Choubert, 1998; atlantic salmon *Salmo salar*, Christiansen & Torrissen, 1997; Trinidadian guppy *Poecilia reticulata*, Grether *et al.*, 2008). Thus, there is evidence that effects of carotenoids might be more taxon- (Grether *et al.*, 2008) or context-specific (e.g. rainbow trout, see above).

6. Carotenoid-based ornaments as honest quality indicators?

Most studies investigating the carotenoid trade-off hypothesis deal with species where males and/or females show carotenoid-based ornaments - the differences in intensity of carotenoid-based signals making different carotenoid investment

easily detectable. It has been argued that the carotenoid-based coloration is an honest indicator of quality (Milinski & Bakker, 1990; Hill, 1991; Godin & Dugatkin, 1996). Generally, the handicap principle suggests that signals which are costly to produce are reliable indicators for quality in the long term because they cannot be faked (Olson & Owens, 1998). The trade-off in carotenoid investment between ornament and immune function or antioxidant activity makes carotenoid based ornaments a potential candidate for honest signals, with only the high quality individuals being able to maintain carotenoid investment into coloration under conditions of carotenoid scarcity. According to the "good genes" or "viability indicator" model, a correlation exists between genetic quality and viability, and the female benefits from mating with an attractive i.e. high quality male (quality being possibly advertised through ornamentation) by increasing the viability of her offspring (Kokko, 2001). This prediction has been verified in numerous studies. In the peacock *Pavo cristatus* offspring from males with more elaborate ornamentation of the tail feathers are bigger which results in higher subsequent chance of survival to adulthood (Petrie, 1994). In the great tit *Parus major* females preferentially mate with males showing large black breast stripes, a feature which is inherited by their sons and positively correlated with the survival of the male offspring (Norris, 1993). In a Trinidadian guppy population females prefer to mate with larger males which were shown to sire offspring with higher growth rate which results in bigger sons (that themselves have higher mating success) and a positive effect of size on daughters' reproductive output (Reynolds & Gross, 1992). In another population of the same species, the percentage of the body covered by yellow, orange or red spots which contain carotenoids has been shown to be positively correlated with the ability to escape a simulated predator attack (Evans *et al.*, 2004). In the house finch *Carpodacus mexicanus* males exhibit a plumage coloration which can range from pale yellow to bright red. It has been shown that in this species, plumage coloration reflects the type and quantity of carotenoid ingestion and is thus a function of foraging ability. Females prefer the reddest male, and more intensely colored males do not only show enhanced feeding behavior directed towards the female and the offspring alike (direct benefit) but also show higher survival rates during winter when compared with drab males. This is a hint for a better genetic quality of the redder males (indirect benefit) which is likely

passed on to the offspring as sons of intensely red males were found to show an intense plumage coloration as well (Hill, 1991). Thus, ornaments based on carotenoids have been shown to honestly advertise mate quality in some species.

7. Egg ornaments in focus

Recently a number of studies investigated the hypothesis that egg pigmentation might have a function as an ornament, altering the traditional view that predation or brood parasitism are the main forces shaping egg coloration. Avian eggshell color is very variable among but also within species (Morales *et al.*, 2011) and it has been suggested that males of species with biparental care use egg coloration to assess the reproductive value of the clutch and thus make decisions about their parental effort (Morales *et al.*, 2010). The sexually selected eggshell coloration hypothesis (SSEC) predicts that biliverdin-based blue-green eggshell coloration is an indicator of female and/or nestling quality and was supported by some studies (Soler *et al.*, 2005; Cherry & Gosler, 2010). The possible mechanism behind this is that the blue-green egg coloration is a signal of female antioxidant capacity (Moreno & Osorno, 2003). Biliverdin pigment possesses important physiological functions similar to carotenoids (Morales *et al.*, 2011) and as in carotenoids, the female might face a trade-off between investment of biliverdin into antioxidant defences versus eggshell coloration. These assumptions are met in Gray Catbirds *Dumetella carolinensis* where females with higher total antioxidant capacity lay eggs with higher blue-green chroma. Males provide more care to nestlings from clutches with higher average blue-green egg chroma (Hanley *et al.*, 2008). Similar results were found in the American robin *Turdus migratorius*. When given artificial eggs either vividly or pale colored which were later replaced by foster-nestlings, males showed a higher provisioning rate towards nestlings that had replaced intensely colored eggs when compared with the pale egg treatment or untreated controls. However, this difference in paternal effort was only visible during the first few days of nestling development and disappeared in later stages (English & Montgomerie, 2011). More investigations about the reasons for the presence of biliverdin in the eggshell will have to be done to really clarify its' function (reviewed in Reynolds *et al.*, 2009). Nevertheless, similarities might exist between the function of biliverdin in bird eggs and carotenoids in fish eggs, especially

because of their similarities in physiological properties (antioxidant activity). In fish species where males show some kind of brood caring behaviour, they might use the intensity of egg coloration to make decisions about their paternal effort. With no solid eggshell being present in fish, differential carotenoid investment of the female into her eggs would be easily detectable for the males judging from the egg coloration and could be a hint for female but also the offspring quality. Egg coloration in context with carotenoids has hardly been looked at in detail so far, as it is usually only egg carotenoid content which is investigated. There are few studies mentioning the possible connection of egg color and carotenoid supplementation (Tyndale *et al.,* 2008) and even less studies explicitly investigating the correlation between carotenoid content and the color of the egg (Harris, 1984; Verakunpiriya *et al.,* 1997). However, as mentioned above, egg color could be one of the criteria on which parents base the magnitude of their parental effort, if it advertises offspring quality.

8. Sexually antagonistic genes

It has been argued that there might be differences in the offspring's inherited genetically-based fitness benefits related to offspring sex. The majority of genes are not sex-specific, thus providing a possible basis for conflict (Fedorka & Mousseau, 2004). This sexual antagonisms might be most influential where the sexes diverge the most, namely in reproduction (Foerster *et al.*, 2007). In *Drosophila melanogaster* genomes promoting greater egg production in females reduce fertilization success in males and vice versa (Chippindale *et al.*, 2001). In the cricket *Allonemobius socius* paternal mating success is negatively correlated with female offspring's reproductive output but positively correlated with male offspring's mating success (Fedorka & Mousseau, 2004). In song sparrows *Melospiza melodia*, extra pair female offspring were less likely to survive to independence and lived shorter than within pair female offspring, while the opposite effect was found for male offspring (Sardell *et al.*, 2011). In the dung beetle *Onthophagus Taurus*, sire effects were found on the males' survival to sexual maturity, while this was not found in female offspring (Garcia-Gonzalez & Simmons, 2011). Exaggerated mandibles are expressed in broad-horned flour beetle (*Gnatocerus cornutus*) males and used for male-male combat while this trait is not expressed in females. Nonetheless, females from populations selected

for larger male mandibles have lower fitness, whereas females in small mandible populations have highest fitness (Harano *et al.*, 2010). The existence of long tail feathers (a trait correlated with higher reproductive success in males) in female barn swallows *Hirundo rustica* seems to be only a product of genetic correlation between sexes and no positive influence of longer tail feathers on female reproductive characteristics can be observed (Cuervo *et al.*, 1996). In the common side-blotched lizard *Uta stansburiana* males exhibiting an orange throat and high antibody responses showed an increased survival, while the combination of those two traits in females reduced their fitness (Svensson *et al.*, 2009a). In the three-spined stickleback the expression of red pelvic spines, a typical male trait, is a disadvantage for the females showing it, since males prefer to direct courtship behaviour towards females with drab and not ornamented spines when given the choice (Nordeide, 2002). Studies explicitly investigating the possible genetic correlation between the capacity of a stickleback male to invest into his carotenoid-based breeding coloration and the capacity of his daughter to invest carotenoids into her eggs are lacking so far.

9. Carotenoids in a challenging environment

Dietary antioxidants are especially important for the organism when exposed to acute stressors resulting in a disbalance between the oxidants and antioxidants necessary for maintaining normal physiological function, which can no longer be fulfilled by the endogenous antioxidants only (Edeas, 2011). There are numerous studies investigating the positive effects of antioxidants after the organism is challenged by some kind of stressor. For example, treatment with vitamin E was found to prevent impairment of long- and short-term memory in rats as a consequence of chronic sleep deprivation through its antioxidant function in the hippocampus (Alzoubi *et al.*, 2012). Supplementation of vitamin E led to an improvement of feed intake, body weight gain, feed efficiency, egg production and quality, nutrient digestibility, immune response and antioxidant status in poultry birds exposed to heat stress, which is characterized by reduced antioxidant status resulting in oxidative stress (Khan *et al.*, 2011). In Pacific white shrimp, the enzymatic antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase in the muscle and pancreas responded in very specific manner to conditions of environmental hypoxia followed by

reoxygenation (which increases the number of ROS) and thus prohibited oxidative damage in the organism (Parrilla-Taylor 2011). In the Eurasian kestrel, offspring of females supplemented with extra carotenoids were less susceptible to intestinal parasites (De Neve *et al.*, 2008).

In the study of carotenoid actions in fish, the impact the environment and environmental factors challenging the organism's immune system might have on finding benefits of carotenoids, has so far not been focused on much. Many studies investigating the effects of carotenoid supplementation were done with commercially bred species (e.g. rainbow trout, salmon species) and it is usually the potential enhancement by carotenoids of reproductive output, health, longevity, flesh pigmentation and immune responses in vitro which is studied (e.g. Bjerkeng *et al.*, 1990; Christiansen & Torrissen, 1997; Verakunpiriya *et al.*, 1997; Choubert, 1998; Amar *et al.*, 2000; Amar *et al.*, 2001; Ahmadi *et al.*, 2006; Lakeh *et al.*, 2010). However, fish kept in fishery institutions or under sterile laboratory conditions possibly experience a much less "dangerous" environment than their wild conspecifics in terms of possible stressors. Thus the significance of carotenoid actions might so far have been underestimated in fish and tests under a more natural environment might be crucial for estimating the impact of carotenoids. A discrepancy between studies in the laboratory and the wild has for example also been found when testing endocrine-disrupting chemicals: laboratory studies sometimes fail to detect effects that are present under more realistic ecological conditions (reviewed in Zala & Penn, 2004). The same might be true in studies on carotenoid effects.

10. The three-spined-stickleback

In the three-spined stickleback males show a typical, partly carotenoid-based breeding coloration with intensely red colored throat and blue eyes (Brush & Reisman, 1965; Wedekind *et al.*, 1998). In this species the males build nests out of plant nesting material into which several females lay their eggs and the male cares for the brood until one week after hatching (Kynard, 1978; Wootton, 1984). Brood caring behavior (e.g. fanning the nest) is energetically demanding (Chellappa *et al.*, 1989). More intensely red colored males are preferred by females over duller ones (reviewed in Bakker & Milinski, 1993). Red coloration has been found to be an indicator of male quality in several studies: parasitation

decreases intensity of red coloration and females can use this information to avoid mating with parasitized males (Milinski & Bakker, 1990). More intensely colored males were in better condition in laboratory studies (Bakker & Milinski, 1993; Candolin, 1999), which was confirmed for at least one wild population (Bakker & Mundwiler, 1994). Expression of red nuptial coloration was found to be positively correlated with male fertilization success and life span (Pike *et al.*, 2007a; Pike *et al.*, 2009). Furthermore, a negative effect of paternal redness on the number of offspring becoming infected by an experimentally introduced cestode parasite was reported by (Barber *et al.*, 2001). Redder fathers produce on average significantly redder sons, indicating an additive genetic variation for red intensity. Furthermore a positive genetic correlation between the fathers' ornament and the daughters' preference for male nuptial coloration is present in the species (Bakker, 1993b). Thus, by choosing the more intensely red colored male, the female gains indirect benefits enhancing her offspring's' viability.

In sticklebacks only the male provides parental care for the eggs/hatchlings and limitations might be present concerning the male's sperm supply and the number of eggs/clutches one male can care for at a time (Zbinden *et al.*, 2001). Thus, in the case that ripe females are abundant, there might be criteria by which males decide whether to "accept" a clutch from a certain female or not (Nordeide, 2002). However, while it is known that stickleback males prefer larger females and females with a more distended abdomen - both traits indicating egg size or egg number produced by the female (Rowland, 1982; Sargent *et al.*, 1986; Fletcher & Wootton, 1995; Kraak & Bakker, 1998), male mate choice in context with female ornamentation is poorly investigated in the species. Interestingly, female carotenoid-based ornamentation has been found in some stickleback populations. In a stream-resident population in British Columbia, many females develop orange-red throat coloration markedly different from the coloration of the females of a nearby anadromous population and resembling more the red breeding coloration of a stickleback male. Some females were found to be as intensely colored as the males. However the distribution of the red ornament was typically less in females. When the males of the stream-resident and anadromous populations were compared, no significant differences in breeding coloration were found, thus suggesting that female ornamentation is not a by-product of the evolution of male coloration.

Since the red coloration of the stream-resident females is not enhanced during ovulation, the ornamentation is unlikely to be a signal of short-term reproductive readiness (McKinnon *et al.*, 2000). Females of a stream-resident stickleback population in Northern California also have been reported to exhibit red coloration on their throats, although less intense and distributed as the coloration of a breeding male of the same population (von Hippel, 1999). In several European stickleback populations, not only males but also females show carotenoid-like reddish coloration on the inner side and the basis of their pelvic spines (Nordeide, 2002; Frommen, personal communication). When males of one of these populations were presented with a female showing red pelvic spines and a female with drab spine coloration, the courtship activity directed towards the drab females was significantly higher than towards females with red ornamented spines. However, this preference disappeared when females were presented under green light, making it impossible for the males to detect differences in red ornaments. Thus, the reddish color of the females' spines seems to have a signalling value for males and even if not aimed at the males in the first place, it has been shown to have a potential impact on sexual selection (Nordeide, 2002). Summarizing, carotenoids and carotenoid investment into ornaments do not only seem to play a role in stickleback males but also in females. In populations where female red ornamentation occurs, carotenoid based signals might be important for both sexes in the context of reproduction. However, further investigations with stickleback populations showing ornamented females are necessary to clarify the significance of female coloration and its evolutionary background.

Stickleback females are known to lay clutches differing in egg coloration (see *Appendix,* 6.1). Some clutches consist of eggs being uncolored and transparent, others show a very intense yellowish coloration, and some clutches are intermediate (personal observation). Color variability within a clutch appears low compared to variability between clutches. Although carotenoid investment into eggs and its influence on offspring fitness parameters has been investigated in some fish species (Christiansen & Torrissen, 1997; Tachibana *et al.*, 1997; Verakunpiriya *et al.*, 1997; Amar *et al.*, 2004; Ahmadi *et al.*, 2006; Grether *et al.*, 2008; Garner *et al.*, 2010; Lakeh *et al.*, 2010), no such investigation has ever been conducted in three-spined sticklebacks despite the broad knowledge about

the importance of carotenoids in sexual selection of the species. Additionally, although there is evidence that redder fathers sire redder male offspring (Bakker, 1993b), the question if there also is a link between a father's carotenoid-based breeding coloration (showing his carotenoid investment capacities) and his daughters' capabilities of carotenoid use, possibly visible through the daughters' investment of carotenoids into their eggs, has never been investigated yet. Sexually antagonistic genes might be present, thus making it an advantage to have an intensely colored father for the male offspring, but a disadvantage for the females. Alternatively, both sexes might benefit from having a father with high carotenoid investment capacities, or the investment of carotenoids into eggs in the female offspring generation might not be linked to any carotenoid-mediated trait in the father.

In this study I investigated the following battery of questions using the three-spined stickleback as study organism:

(A) Is there an effect of carotenoid supplementation on maternal investment of carotenoids into the egg and if so, does different carotenoid investment lead to differences in the viability of the offspring in terms of hatch/survival rate and growth rate? Do carotenoids have a positive, negative or no effect for the offspring's viability? Does egg coloration reflect the availability of carotenoids in the mother's diet? Is there an influence of rearing environment on the effects of carotenoids?

(B) Do high quality males which have been shown to exhibit an intense carotenoid-based breeding coloration not only pass the ability of carotenoid investment into ornaments on to their sons but also to their daughters? Is there evidence for the existence of sexually antagonistic genes or is the relationship between the carotenoid investment ability of a male and the one of his female offspring of another kind?

11. Expectations

(A) Based on the information presented above, it seems likely that in sticklebacks, carotenoid supplementation of females can have an influence on the carotenoid content of the egg and - as a consequence - on egg coloration. If any, I expect carotenoids to have a beneficial effect on the viability of the

stickleback offspring on the background of the numerous studies reporting positive effects of carotenoids in other species. By my experimental design of raising groups of siblings in environments differing in the amount of pathogens they contain, I aim to broaden the knowledge about carotenoid effects in vivo under challenging conditions for the immune system. Concerning the possible effect maternal carotenoid supplementation might have on offspring viability in a low and a high pathogen environment, there are different scenarios which can be imagined. On background of findings of previous studies investigating the importance of carotenoids in early life stages the two processes most likely are the following:

(1) In a low pathogen environment, eggs containing few carotenoids might have little risk of reduced viability in comparison to eggs containing many carotenoids. This might be different in the high pathogen environment, in which eggs containing few carotenoids might suffer a cost compared to carotenoid-rich eggs, which benefit from the positive effects carotenoids have when the immune system is challenged (Figure 1, scenario 1, white circles represent low pathogen, green circles high pathogen environment).

(2) Low carotenoid content of the egg might have a negative effect on offspring viability, independent of the environment the offspring is raised in. Offspring from eggs rich in carotenoids would then show higher fitness compared to eggs containing few carotenoids whether the immune system is challenged or not (Figure 1, scenario 2, white circles represent low pathogen, green circles high pathogen environment).

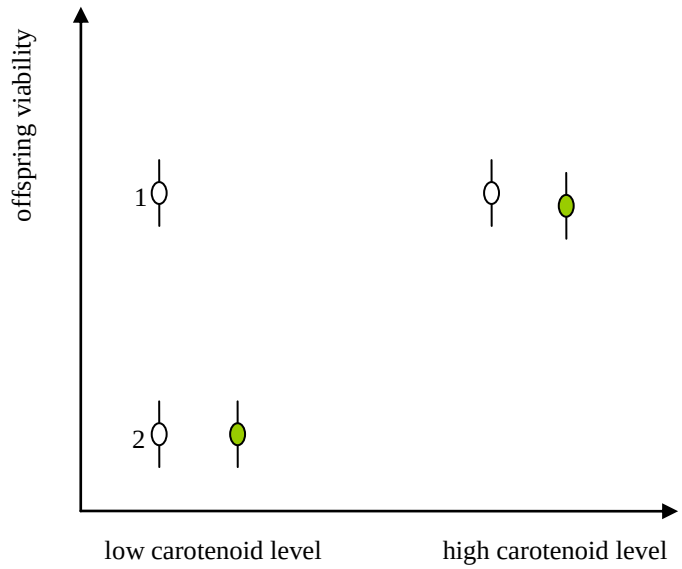


Figure 1. Two possible scenarios for the effect of carotenoid supplementation on offspring viability in a low versus a high pathogen environment.

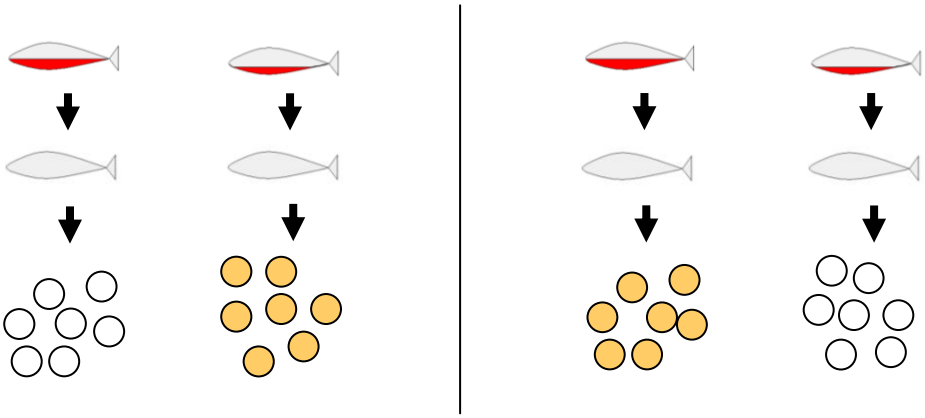
So far, effects of carotenoid supplementation in fish have mainly been studied in the same few species, which are often fish kept for commercial use in fishery institutions. These fish might live under totally different environment than fish in the wild (e.g. clean water, prophylactical disease control). Mimicing a more natural environment when investigating the effects of carotenoids might be crucial to detect relevant results. A direct comparison between offspring raised in a low pathogen environment versus a high pathogen environment has never been done before. However such an experimental design might be especially usefull to find out more about the function of carotenoids under stressful conditions. The possible importance of the connection of carotenoid content and egg color in fish has also been widely neglected so far. However, if egg coloration is a good measure for egg carotenoid content and more carotenoids in the eggs are beneficial for the offspring's viability, fish could use "egg ornamentation" for assessing offspring quality, which might be important for decisions on parental investment in brood caring species.

(B) Since male sticklebacks inherit their ability to invest carotenoids into their sexual ornaments during the breeding season from their fathers, it seems likely that the females might inherit the ability to invest carotenoids into eggs. However, it can not be said a priori if genetically antagonistic genes are present

or if both, male and female offspring benefit from having a high quality father, able to invest a lot of carotenoids into his ornament. Thus, again two possible outcomes of the study can be imagined assuming that offspring benefit from a relatively high carotenoid amount present in the eggs:

(1) If sexually antagonistic genes are found, daughters from males showing an intense breeding coloration would produce eggs containing few carotenoids which exhibit a drab coloration. Assuming that a high level of carotenoids is beneficial for the offspring, daughters from high quality males would thus produce low quality offspring (Figure 2, left side, intensity of male breeding coloration is indicated by a big or small area covered with red color).

(2) If no antagonistic genes are present and both sexes benefit from having a high quality father showing an intense red breeding coloration, daughters of such fathers would lay eggs containing a relatively high amount of carotenoids, which would increase the viability of the offspring under the assumption that carotenoids have beneficial effects (Figure 2, right side, intensity of male breeding coloration is indicated by a big or small area covered with red color).



Three-spined stickleback Likely scenarios by which a father's and daughter's ability to invest carotenoids into (breeding or egg) ornaments could be linked. The first line shows the males, the second line the female offspring, and the third line the eggs produced by the female offspring. The left side shows the presence of sexually antagonistic genes, while the right side presents the outcome if both offspring sexes benefit from having a father able to invest a lot of carotenoids into his nuptial coloration.

Part A: The effect of maternal carotenoid supplementation on egg characteristics and offspring fitness parameters in the three-spined stickleback

A.1 Material and Method

A.1.1 Study population

Adult three-spined sticklebacks were caught on the island of Texel in the Netherlands during their spring migration in April 2010. They represent a large, genetically heterogeneous (Heckel *et al.*, 2002) stickleback population. In Texel the natural migration routes from the sea to the freshwater breeding grounds have been destroyed by the building of artificial canals. Today sticklebacks are caught in big basins open to the sea, from where they are artificially pumped over the dykes (Kemper, 1995). From the collecting basins a representative sample of the population can easily be taken for scientific purposes. The wild-caught fish were brought to the Konrad Lorenz Institute in Vienna, Austria, where males and females were kept together in 500 and in 1400 liter outdoor tanks before they were used in the experiment. Outside tanks where equipped with an Eheim powerline filter (230V/50Hz) for aeration and flower pots at the bottom serving as hiding places. Fish where fed in excess with frozen bloodworms *Chironomus* spp. once a day.

A.1.2 Experimental design

A.1.2.1 Parental generation

A.1.2.1.1 Maintenance of the females

During the breeding season, males and females can easily be distinguished: males show their typical breeding coloration with a red colored throat and belly and blue eyes (Wootton, 1984). All fish not showing these characteristics at least partly where classified as females. Females used for the experiment were kept in

the laboratory in large 300 and 400 liter tanks in groups of 35 fish per tank at the maximum. Tanks were equipped with a sponge air filter, an additional air stone and two big plastic plants each. Illumination came from lamps installed over the tanks. The light regime was set to summer (breeding) conditions with a 16:8 light:dark cycle without twilight. Room temperature was kept at 18±1°C. Females were fed once a day with a diet based on fish meal, plant oil, and food coloring powder (Co. Schimek, color 4: strawberry-red) following Pike *et al.*, 2007b. To this basic formula, different amounts of carotenoids, kindly provided by DSM Basel, Switzerland, were added (Table 1). Females were divided into three different diet groups corresponding to the doses of carotenoids added to the food. While the control treatment group was not supplemented with carotenoids, food for the other two diet groups contained 20mg/kg food or 100mg/kg food of astaxanthin and lutein each (Table 1). I chose those two carotenoids, because (1) both of them have been found to play a role in the males' breeding coloration (Wedekind et al., 1998), (2) studying gonad carotenoid content revealed that derivates of astaxanthin and lutein were present in female sticklebacks (Nordeide *et al.*, 2006) and (3) all studies testing the effects of carotenoid supplementation in the species have used a combination of at least those two types of carotenoids (Wedekind *et al.*, 1998). From now on the three different treatment groups will be referred to as zero, low and high carotenoid treatment. The three different doses of carotenoids are within the range of the ones usually found in experiments using artificial carotenoid supplementation of fish including sticklebacks (e.g. Bjerkeng *et al.*, 1990; Christiansen & Torrissen, 1997; Verakunpiriya *et al.*, 1997; Choubert, 1998; Amar *et al.*, 2004; Ahmadi *et al.*, 2006; Sawanboonchun *et al.*, 2008; Garner *et al.*, 2010; Lakeh *et al.*, 2010). Since the wild-caught females consumed their food best when they were undisturbed, they where fed on excess once a day in the late afternoon and all food left in the morning was thoroughly sucked off the tanks' bottom with a tube. Water was changed every day.

Table 1. Ingredients for the food of the three different diet groups

Carotenoid treatment	Anchovy meal	Sun flower oil	Red food coloring	Astaxanthin 10%	Lutein 5%
zero	1000g	500g	0.67g	-	-
low	1000g	500g	0.67g	0.2g	0.4g
high	1000g	500g	0.67g	1g	2g

A mixture of anchovy meal, plant oil and red food colouring was used as base compound. Different doses of astaxanthin and lutein were added in order to create food for three different diet treatments.

Before allowing the females to spawn, they were kept on the artificial diet for a minimum of 26 days. During the breeding season, females produce eggs within a few days (Wootton, 1974) and spawn the complete clutch as soon as eggs are fully developed. Females that do not find a mating partner within approximately 24 hours spontaneously spawn their eggs and start producing a new clutch. The average interspawning interval ranges between 2.2 and 7.8 days (Wootton, 1974). Should a female have started egg production before being transferred from the outside tanks into the laboratory and before being put on the experimental diet she spawned those eggs and started production of a new clutch during the feeding with the artificial diet. Thus, it was ensured that the eggs laid by the females used in this study were entirely produced after the females were put on the experimental diet and could not have contained carotenoids derived from the previous diet of *Chironomus* spp.

A.1.2.1.2 Breeding

Breeding was conducted between the 2 June and the 17 July 2010. Gravid females of the three diet groups were allowed to spawn with males kept in the laboratory under the same light and temperature conditions as females. Males were housed individually without visual contact to other males in 12 liter tanks (30x20x20cm) equipped with a petri dish (Ø9cm) filled with sand, 5g of artificial nesting material (4cm pieces of green 100% polyacrylic wool, Co. Schoeller und Stahl, color code: 058, (Burley, 1980) and an air stone. Wool was cooked for a few seconds before being used to remove color remainders. The petri dish was placed at the back of the tank. Water in the breeding tanks was changed once a week. A gravid female was presented to the males for at least five minutes daily to stimulate nest building (e.g., Frommen & Bakker, 2006).

Males that did not respond with nest building behavior by this procedure within three days were given new nest material, and were replaced by a new male if they still did not respond after this. Males were fed once a day with frozen bloodworms (*Chironomus* spp.). Beginning on day 26 after the establishment of the three diet groups, the tanks of the females were scanned for gravid individuals every day. Gravid females where identified by their enlarged abdomen and prominent cloaca, through which the ripe eggs can be seen when gently squeezing the belly. All females ready to spawn were caught and individually put into a male's tank where a nest had been established. To provide the females cover from the males and to ensure that the males directly fertilised the eggs after spawning without being disturbed by the prolonged presence of the female, a green, opaque divider serving as hiding place for the female was added to the males' tanks. This cover was readily accepted (i.e. after successful spawning where the male chases the female from the nest). Gravid females that did not show courtship behavior after being placed into a male's tank were removed and tested with a second and third male. If they did not court at all, they were replaced into their holding tank to reduce stress. The majority of females accepted the first male they were assigned to (personal observation). Males that had spawned were removed within 30 minutes after spawning and put into a separate holding tank to ensure that they where only used once. Females were weighed before and after spawning, and standard length was measured. From these data, condition of the fish was calculated as the residual value of weight x standard length. Female weight after spawning was used for calculations.

As fertilization process in sticklebacks is slow (Bakker *et al.*, 2006) and the chorion needs time to harden (Wootton & Evans, 1976; Kraak & Bakker, 1998; Frommen *et al.*, 2008), the clutches were removed from the nest after two hours had passed post spawning. This procedure ensured that all clutches were removed from the male's nests at same stage of development, in two-cell or four-cell stage as described by (Swarup, 1958). Before taking the eggs out of the nest, males were caught and their weight and standard length was measured. Again condition was calculated from these data in the same manner as for the females.

Whereas every female was only used for one breeding, males were used up to three times, but never twice in the same treatment. Thus, they sired a clutch of a female of each of the three diet treatments. This was done to control for the effect of males' quality on egg and offspring survival. The order in which females of the different diet groups were assigned to the males was randomized. However, only 12 out of 18 males used did successfully sire three clutches. Two males sired two clutches and four males sired only one clutch.

A.1.2.2 F1 Offspring

A.1.2.2.1 Collection of egg data

To collect eggs, the petri dish with the male's nest was removed from the tank in the absence of the male. The eggs were carefully taken from the nest and the petri dish put back into the tank at the same position with the entrance of the nest oriented into the same direction as before egg removal. Afterwards, males were replaced into their tanks. Eggs of every clutch were counted and total number of eggs as well as number of fertilized eggs, which could easily be spotted by the presence of a developing embryo (Swarup, 1958), was recorded. 10 eggs of every clutch were carefully put onto a paper tissue for a few seconds to remove adherent water from the egg surface. Then the eggs were placed on a microscope slide and photographed from above using a Canon EOS 400D camera with an EFS 60mm macro lens. A black piece of non-reflecting fabric was placed under the microscope slide bearing the eggs when being photographed. A white Novoflex Card (see below) and a piece of mm paper for size reference were visible on every picture. Conditions were standardized by placing the microscope slide with the eggs into a photo box lined with black cloth and using a Volpi Intralux 6000 fiber optic light source for illumination. The distance between the camera lens and the microscope slide and the camera settings (aperture and exposure time) were the same for all egg samples. To correct for possible illumination differences between the pictures, the standardized white side of a Novoflex Zebra Grey Card visible on each picture was used as a reference (Bakker & Mundwiler, 1994). Egg coloration was measured in the CIE 1976 (L*, a*, b*) colour space, giving the advantage of being more device independent than the RGB color space (Stevens & Cuthill, 2005). To simplify matters, I will refer to the color space as "*Lab*" from now on.

In *Lab*, *L* values give information about the lightness of the picture, while the *a* axis represents the color spectrum ranging from green (negative values) to magenta (positive values) and the *b* axis represents the range from blue (negative values) to yellow (positive values) (Figure 3).

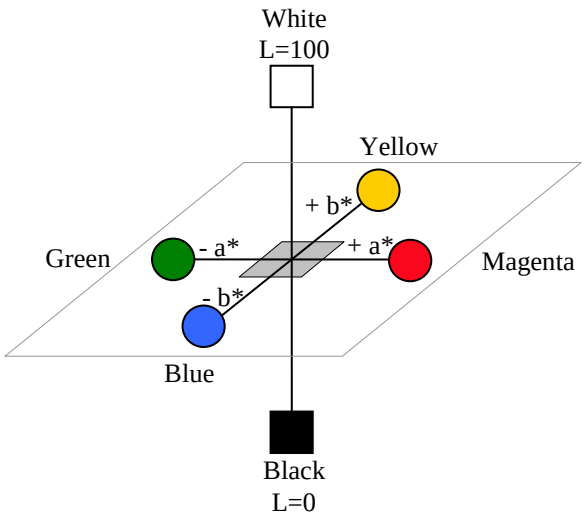


Figure 3. Simplified Illustration of the three axes in the CIE 1976 (L*, a*, b*) colour space. L* represents the lightness of the color in a scale from 0 to 100. The two color axes define the color in the range from green (negative a* values) to magenta (positive a* values) and blue (negative b* values) to yellow (positive b* values).

Egg coloration in *Lab* color space was measured with the color sampler tool of Photoshop CS3, using the average of four measuring points on every single egg to get an average coloration score for every egg (Figure 4). From these average values for each of the 10 photographed eggs, average clutch coloration could be calculated (one average *L* value, *a* value and *b* value for every clutch). Since egg coloration appears much more variable between than within clutches and eggs of one clutch are generally very similar in coloration (personal observation, Figure 18, see *Appendix 6.1*) the method of using 10 eggs to calculate the average egg coloration value for every clutch is acceptable.

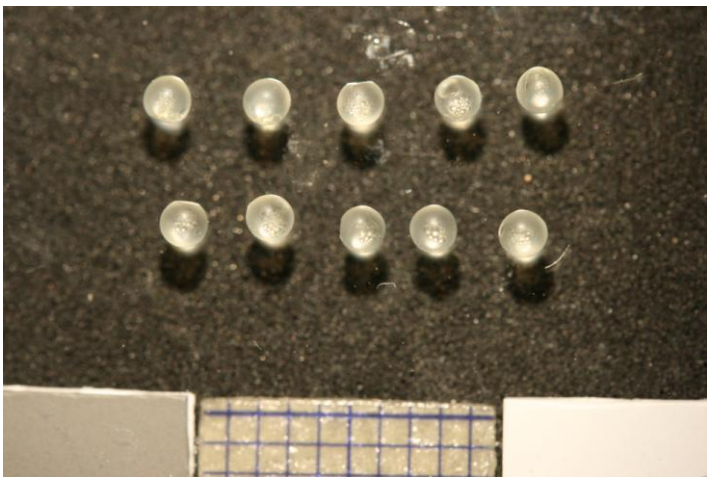


Figure 4. Photograph of 10 fertilized eggs used for egg coloration analysis.

Information about the eggs' lightness was not important in this experiment and the recorded *L* values were therefore not investigated further. To score the differences in egg coloration, the measured average *a* and *b* values were used. These two values were combined to a single "chroma" value (C^*_{ab}) using the formula

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}}$$

in order to get a single coloration measurement for every clutch, "egg chroma".

Egg diameter as a measure of egg size was calculated from the pictures (Treasurer & Ford, 2010) by the reference of a piece of mm-paper visible on every picture. Size measurements were done using the ImageJ program. Single egg diameter was calculated as the average of the horizontal and the vertical diameter to be more accurate. For every clutch, an average egg size value could be calculated from the photographed sample consisting of 10 eggs. After taking the photograph the 10 eggs were weighed together with the slide with a KERN ABS 120-4 weight. Thus, average egg weight could be calculated for every clutch.

A.1.2.2.3 Raising the offspring

During the entire period of data collection, all eggs/hatchlings were kept in a room with 18±1°C room temperature and a 16:8 light:dark cycle without twilight. Eggs that were not used for color and size analysis were split into two

halves of equal size (+/- 1 egg) which were both separately put into a one liter tank equipped with an air stone and raised under different water conditions: one half of the clutch was put into tap water, the other half was raised in water taken from a small pond in the institutes garden. To minimize differences in oxygen content and temperature of tap and lake water, water for both treatments was poured into tanks containing between 300 and 400 liter equipped with an Eheim powerline filter (230V/50Hz) where it stayed at least 18 hours before being used. Water in which the eggs (and later hatchlings) were raised contained 5g of sea salt to reduce the risk of fungal infections (Taylor & Bailey, 1979; Phelps & Walser, 1993; Kitancharoen *et al.*, 1998) as well as one drop of "Nititrkiller" to make the nitrite value of both waters comparable. Water was changed completely every day (Frommen *et al.*, 2008). Dead eggs were counted and removed daily. As soon as the hatching started, the number of hatchlings was recorded daily, as well as the day on which all fish had hatched. The interval between the first and last hatching fish was on average around two days. The hatchlings were fed once a day with live *Artemia* nauplii beginning on the second day after the first hatchling had occurred in a tank. One week after hatching had started, the number of surviving hatchlings of every tank was counted and then reduced to a maximum of 20 fish, in order to minimize differences in growth rate of the hatchlings due to different fish density (Jenkins *et al.*, 1999; Imre *et al.*, 2010). Daily water change and counting of the fish was continued until the fish were 6 weeks old (using the day on which the first hatchling in a tank had been noticed as reference for age). Fish of every tank were photographed every seven days until they were six weeks old, the first picture taken exactly one week after the first hatchling(s) occurred (Figure 5). Photographs were taken with a Canon Eos 400D camera equipped with an EFS 18-55mm lens. Fish were put in a Petri dish with a small amount of water while they were being placed over mm paper and photographed. Only fish of which the whole body was visible in the picture and which held their body axis straight were used for the length analysis with the ImageJ program. The distance of 1cm as defined on the mm paper was measured in pixel. The standard length of the fish was measured in the same way as the straight distance from the tip of the fish's nose to the transition between the caudal peduncle and the caudal fin (i.e. the standard length, SL). Thus, using the number of pixel measured for 1cm on

the mm paper as a reference, the length of the fish measured in pixel could easily be transferred into millimetres as well. In the (few) clutches where less than 10 fish were present, every fish showing a straight body axis on the photograph was analysed for size. In the more numerous clutches, a maximum of 10 fish per picture were measured.

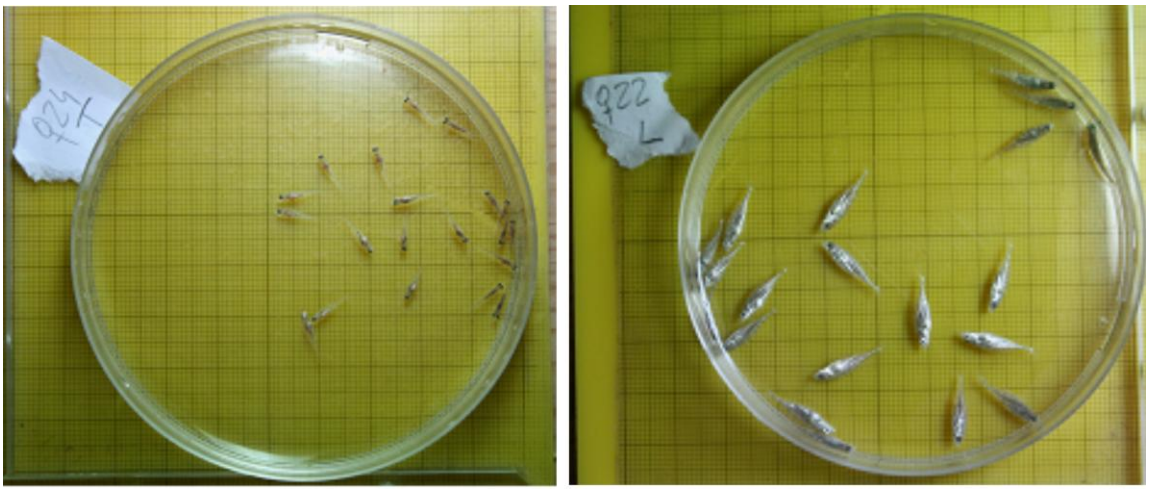


Figure 5. Photographs used for the length analysis of the juveniles at the age of 14 days (left) and 28 days (right).

By these procedures, survival rates as well as growth rates of the fish could exactly be recorded until fish were six weeks old.

A.1.3 Statistical analyses

Differences in the standard length or the condition of the females between treatments: General linear models (GLM) were calculated with standard length or condition as the dependent and female carotenoid diet treatment as fixed factor.

Effect of female carotenoid diet treatment on clutch size and egg size: GLMs were run with clutch size or egg size as dependent variables and carotenoid diet treatment as fixed factor. Female standard length (SL), female condition, date breeding (sooner or later in the breeding season) and – depending on the dependent variable investigated – clutch size or average egg size were added as covariates. Following the GLMs, likelihood ratio tests were run in which the possible explanatory variables mentioned above were individually tested against the null model. In all further cases where the same procedure was used, the

results of the GLMs will be presented mainly in the *Appendix*, while the results of the likelihood ratio tests are explained in detail in the *Results* part.

Effect of carotenoid diet treatment on egg coloration: A GLM was calculated with egg coloration as the dependent variable and diet treatment as fixed factor. Female SL, female condition, date breeding, clutch size and average egg size were included as covariates. Again likelihood ratio tests were conducted for the covariates. Tukey post hoc tests were done to test for possible differences in coloration between the eggs of the females of the different diet groups: zero versus low, zero versus high and low versus high carotenoid dose.

Effect of female carotenoid diet treatment on fertilization rate: Fertilization rates (recorded as the fertilized percentage of eggs of a clutch) were arcsine transformed before being used for statistics in order to reach normality. A linear mixed-effects model (LME) was calculated with carotenoid diet treatment as fixed factor and clutch size, average egg size and date breeding as covariates. Male identity (ID) was included as random factor in the analysis because most of the males (n=12) were used three times. The rest of the males sired two (n=2) or one (n=4) clutch. Likelihood ratio tests were done for female treatment, the covariates and the male effects.

Effect of female carotenoid diet treatment and water quality on hatch rate: Hatch rates were arcsine transformed to reach normality. One outlier, a female which produced a clutch with zero hatching success due to being bred very late in the season, was excluded from the analysis. Another female had to be excluded because a part of the eggs was lost by accident and hatch rate could thus not be calculated. The exclusions did not change the results qualitatively. Since most of the males fertilized more than one clutch, female identity was nested within male identity. The LMEs tested female diet treatment and water quality the offspring were raised in (i.e. tap or lake water) as fixed factors and the interaction between those two variables. Clutch size, average egg size and date breeding were added to the model as covariates. Likelihood ratio tests were done to further investigate the influence of the individual explanatory variables on hatch rate. As water quality had a significant influence on hatch rate (see

results) additional LMEs and likelihood ratio tests were used to analyse the influence of carotenoid diet treatment of the mother on offspring hatch rate in tap water and lake water separately. For the cases in which the likelihood ratio test revealed a significant influence of dietary treatment on hatch rate, Tukey post hoc tests were done to investigate those differences by comparing the hatch rate of the zero versus low, zero versus high and low versus high carotenoid diet treatment groups. Likelihood ratio tests were also conducted to test for female or male random effects on hatch rate.

Effect of female carotenoid diet treatment and water quality on survival of the fish during the first week after hatching: To investigate whether the offspring from the different carotenoid diet female groups were different in their probability of surviving during their first week of life or if possible differences in the survival rates were just reflecting differential hatch rates, a Kruskal Wallis test was conducted which compared the six different raising conditions: offspring from the zero, low, or high carotenoid diet females reared in tap water or lake water. The Kruskal Wallis test was chosen because data on the survival of the fish were not normally distributed. The same two females as mentioned in the last paragraph were excluded from the analyses on offspring survival during the first week of life. After the preliminary statistics, the same tests as for hatch rate were conducted for survival rate.

Female traits influencing the offspring's' hatch and survival rate: Female SL, female condition, clutch size and average egg size were included as covariates in the GLMs investigating the females' influence on hatch or survival rate.

Effect of female carotenoid diet treatment and water quality on offspring size six weeks after hatching: Sample size was reduced to 41 for this and all the following tests, because in three females, all offspring died before reaching the required age. Since the clutch of every female was split into two halves and one raised in tap, the other one in lake water, offspring tanks were nested within female ID for this analysis. Again, as males fertilized more than one clutch, female ID was nested within male ID. A LME was calculated with average

hatchling size as the dependent variable and female carotenoid diet treatment, water quality and the interaction between those two variables as fixed factors. Average egg size, date breeding and the density of the fish in the tank (maximum 20 fish) were included in the model as covariates. Likelihood ratio tests were conducted testing each of the explanatory variables against the corresponding null model. As for hatch and survival rate, LMEs were repeated for tap and lake water separately, all other included variables staying the same. The likelihood ratio test was also repeated for detailed testing of a possible influence of carotenoid diet treatment on offspring size in tap or lake water each. If treatment was found to be influential on size, Tukey post hoc analyses were done to reveal the differences between groups: zero versus low, zero versus high and low versus high carotenoid diet treatment.
Likelihood ratio tests were conducted to test for female or male random effects on offspring size six weeks after hatching.

Effect of female carotenoid diet treatment and water quality on offspring growth parameters: To deeper investigate the impact of the raising environment and of carotenoid treatment of the mother on offspring's growth, several growth models were tested using Akaike information criteria (AIC, see *Appendix* for tested models). The variables included in all the tested models were initial size (*a*), initial growth rate (*b*), growth rate change (*c*) and time factor (*t*). The formula best in describing offspring size was:

$size = a + bt + ct^2;$

To go on with the analysis, individual estimates with the parameters of the model found to be most appropriate were fitted for each raising tank using the function of nls in the nlm library.
Statistical calculations were done on the influence of initial growth rate and growth rate change in the same manner as for hatch and survival rate. In the LMEs, female treatment, density of the fish in the tank and date breeding were used as explanatory variables.

Female traits influencing the offspring's' size, initial growth rate (*b*) and growth rate change (*c*): A GLM was calculated to detect which female variables influenced the size of the offspring at six weeks after hatching. Female variables included in the model were standard length, condition, clutch size, average egg size and egg coloration (chroma). The same tests were calculated for initial growth rate and growth rate change.

A.2 Results

Females (n=44) of the three different carotenoid diet groups (zero, low or high carotenoid level) did not differ in standard length (GLM, $F_{1,42}$=0.003, t=-0.052, P=0.959). Similarly, no differences between the females of the three diet groups could be found in condition (GLM, $F_{1,42}$=0,769, t=0.877, P=0.386).

A.2.1 Clutch size

Clutch size did not differ between carotenoid diet groups (Log Likelihood tests, Table 2). Breeding date, i.e. early or late in the breeding season had no influence on clutch size (Log Likelihood tests, Table 2). However, clutch size was found to be influenced by the SL and the condition of the females as well as the average egg size (Log likelihood tests, Table 2). Females with a higher SL and females in better condition produced bigger clutches (Table 2, Figures 6 and 7), which contained smaller eggs (Table 2, Figure 8).

Table 2: Results of the log likelihood tests investigating the variables influencing clutch size (n = 44).

Explanatory variable	Δ d.f.	χ^2	P
Carotenoid diet group	1	0.081	0.776
Female standard length	1	6.159	0.013*
Female condition	1	4.984	0.026*
Date breeding	1	0.190	0.663
Average egg size	1	8.606	0.003*

Results reaching the significance level of P<0.05 are marked with a *

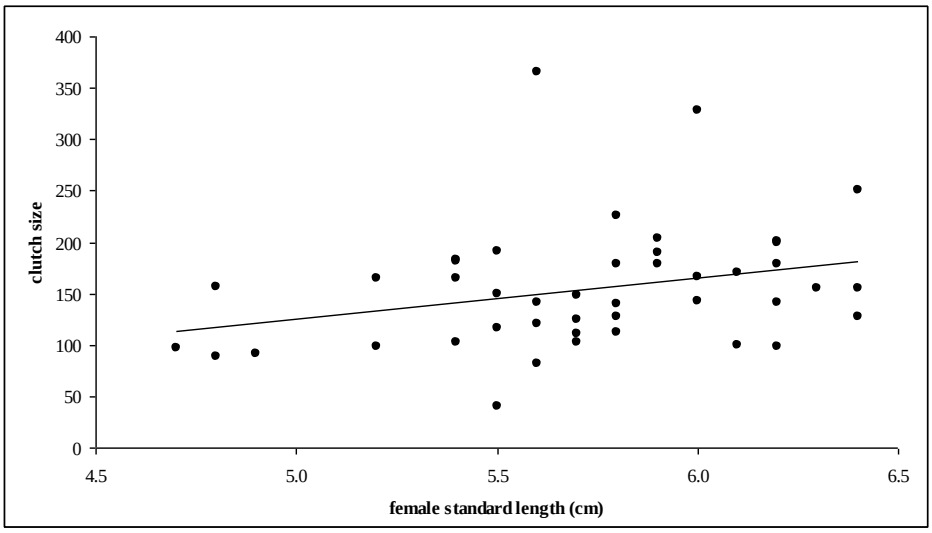


Figure 6. Relationship between female size and clutch size. See text for statistics.

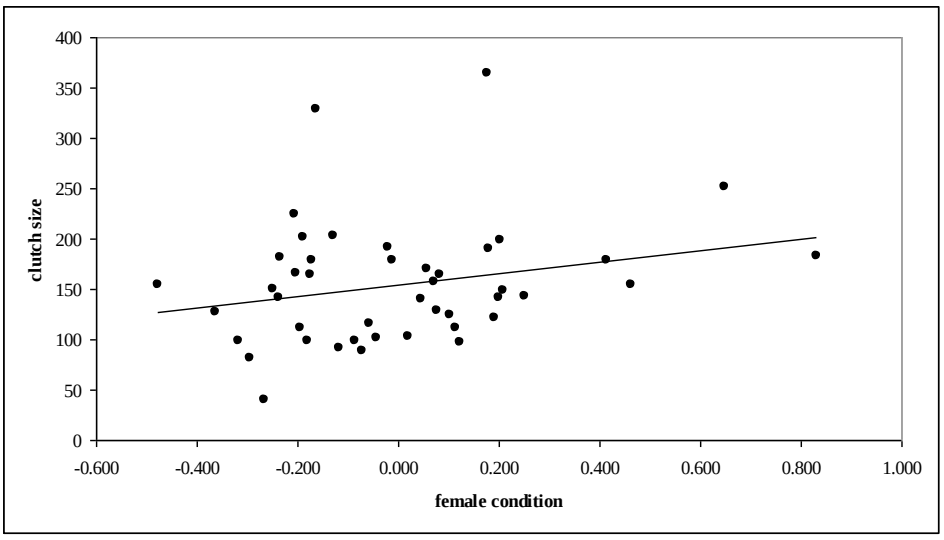


Figure 7. Relationship between female condition and clutz size. See text for statistics.

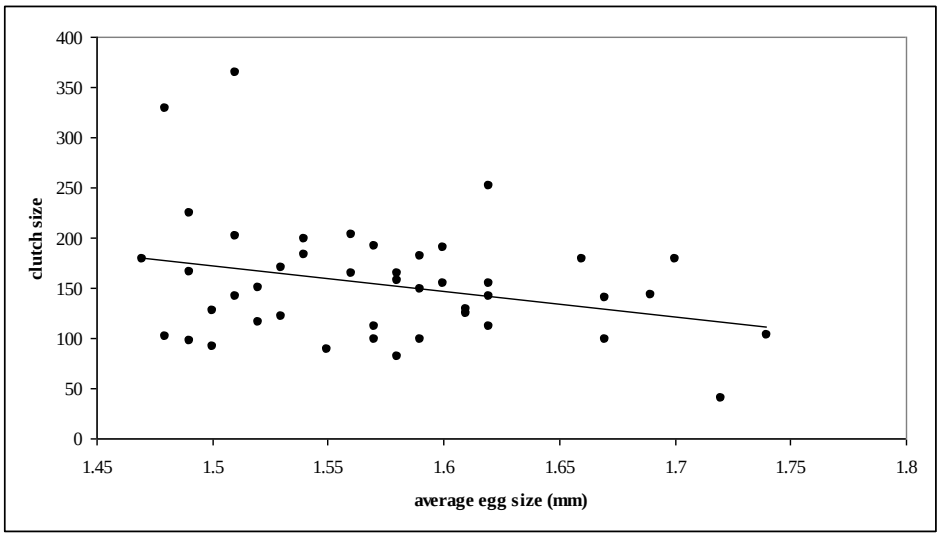


Figure 8. Relationship between egg size and clutch size. See text for statistics.

A.2.2 Egg size

No influence of female diet treatment, female condition or the time in the breeding season the clutch was produced was found on egg size (Log likelihood tests, all $P > 0.073$, Table 3). However, female SL influenced average egg size with bigger females producing bigger eggs (Tab 3, Figure 9). Again, the negative relationship between egg size and clutch size was found (Table 3, see A.2.1, Figure 8).

Table 3: Results of the log likelihood tests investigating the variables influencing average egg size ($n = 44$).

Explanatory variable	Δ d.f.	χ^2	P
Carotenoid diet group	1	0.103	0.748
Female standard length	1	3.863	0.049*
Female condition	1	3.211	0.073
Date breeding	1	0.286	0.593
Clutch size	1	8.606	0.003*

Results reaching the significance level of $P<0.05$ are marked with a *

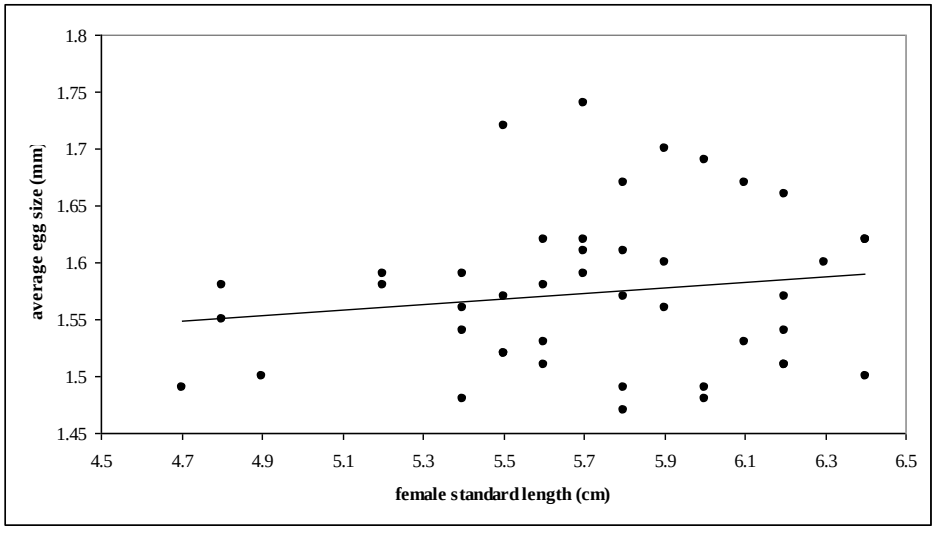


Figure 9. The influence of female size on egg size. See text for statistics.

A.2.3 Egg coloration

Egg coloration was significantly influenced by the diet treatment the female received (GLM, $F_{6,37}=4.125$, $t=4.322$, $SE=1.0365$, $P<0.001$, Table 4). None of

the investigated covariates had a significant impact on the color of the eggs (GLM, all P > 0.145, Table 4). Further investigations about the effect of dietary treatment on egg coloration revealed that the eggs laid by females receiving a diet without carotenoids were not significantly different in coloration from eggs produced by females receiving a low carotenoid level diet, while egg color from both of the mentioned groups differed significantly from the coloration the eggs of the females receiving a high carotenoid diet (Tukey post hoc tests, Table 5, Figure 10). Eggs from the high carotenoid diet females were more intensely colored as the eggs from the other two dietary groups (Figure 10).

Table 4: Results of the general linear model investigating the variables influencing egg coloration (n = 44).

Explanatory variable	*df*	*t*	*P*
Carotenoid diet group	6,37	4.322	< 0.001*
Female standard length	6,37	- 0.366	0.717
Female condition	6,37	- 0.885	0.382
Date breeding	6,37	0.863	0.394
Clutch size	6,37	- 1.490	0.145
Average egg size	6,37	0.393	0.696

Results reaching the significance level of P<0.05 are marked with a *

Table 5: Results of the Tukey post hoc tests investigating the differences in egg coloration (C^*_{ab}) between females of the different carotenoid diet treatment (n=44).

	Δdf	*F*	*P*
Zero carotenoid diet vs low carotenoid diet	1	0.235	0.633
Zero carotenoid diet vs high carotenoid diet	1	13.143	0.002*
Low carotenoid diet vs high carotenoid diet	1	15.022	0.001*

Results reaching the significance level of P<0.05 are marked with a *

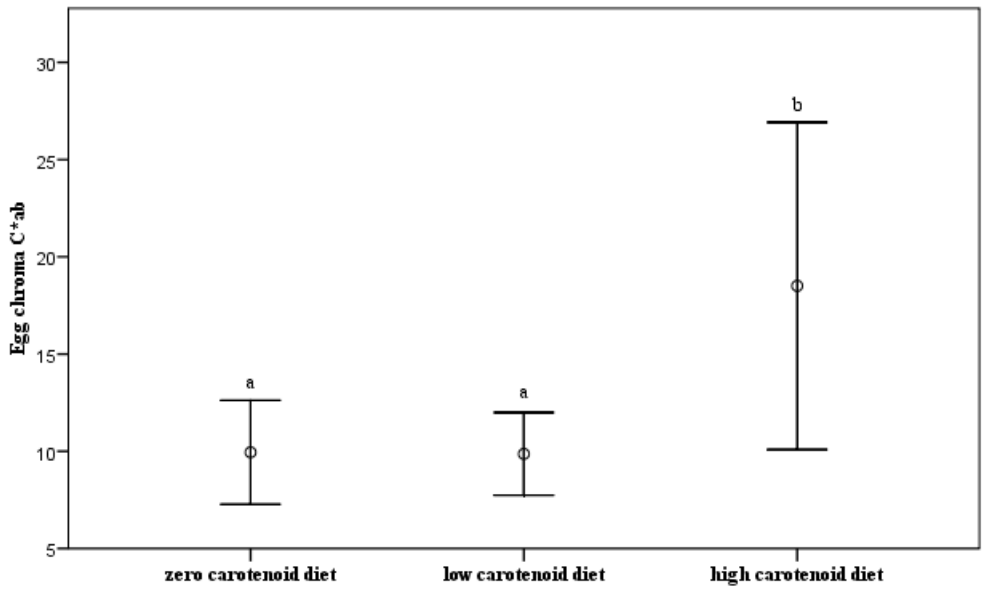


Figure 10. Mean egg coloration (± SD) measured as egg chroma C^*_{ab} of the three different carotenoid diet groups. Significant differences can be recogniced by the bar annotations. See text for statistics.

A.2.3 Fertilization rate

Fertilization rate was not influenced by carotenoid diet group, and no male effects on fertilization rate could be detected (Log likelihood tests, all P>0.385, Table 6). Additionally, none of the investigated covariates influenced fertilization rates (Log likelihood tests, all P>0.354, Table 6).

Table 6: Results of the log likelihood tests investigating the variables influencing fertilization rate (n = 44).

Explanatory variable	Δ d.f.	χ^2	P
Carotenoid diet group	1	0.756	0.385
Male identity	1	$6.504*10^{-9}$	>0.999
Clutch size	1	0.859	0.354
Average egg size	1	0.049	0.825
Date breeding	1	0.069	0.793

A.2.4 Hatch rate

An effect of the interaction between female diet and water quality (tap or lake water) was found on hatch rate (Log likelihood tests, Table 7). Therefore the effect of female treatment on hatch rate was investigated separately for tap and

lake water in the later analyses. Concerning the covariates, hatch rate was not found to be influenced significantly by average egg size or the time in the season the clutch had been laid, but it sank as clutch size rose (Log likelihood tests, Table 7).

Table 7: Results of the log likelihood tests investigating the variables influencing hatch rate (n = 42).

Explanatory variable	Δ d.f.	χ^2	P
Female carotenoid diet × water quality	1	5.788	0.016*
Clutch size	1	4.710	0.030*
Average egg size	1	2.722	0.099
Date breeding	1	2.049	0.152

Results reaching the significance level of P<0.05 are marked with a *.

A. 2.4.1 Hatch rate in tap water

No effect of the level of carotenoid diet was found on the eggs' hatch rate in tap water (Log likelihood test, Δ *d.f.*=1, χ^2=0.415, P=0.520, Figure 11).

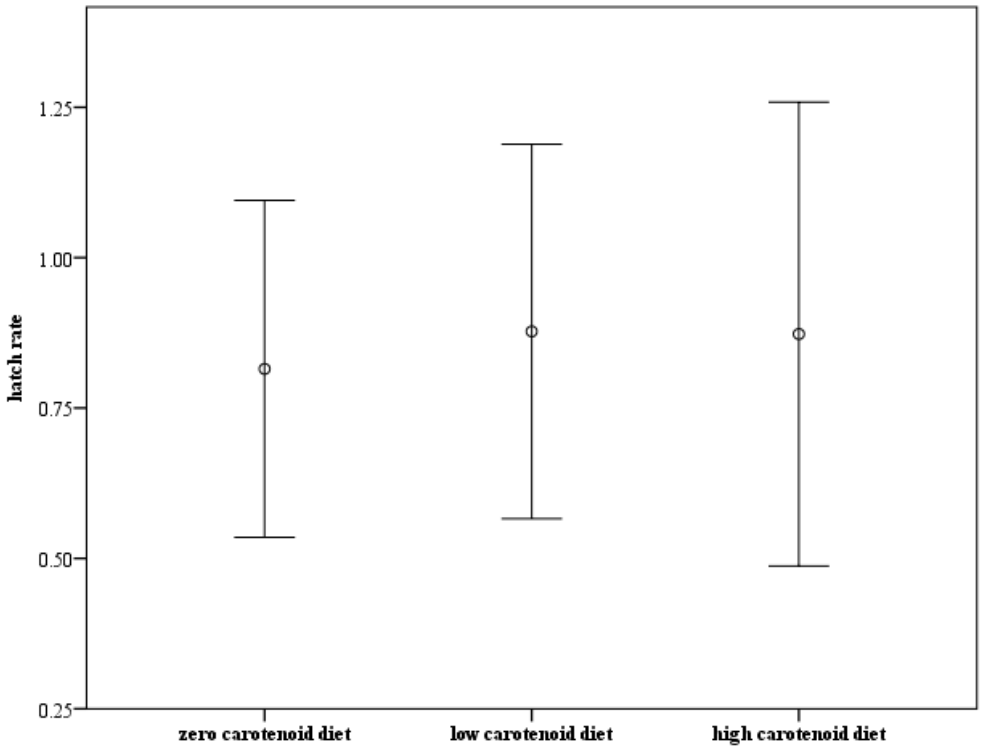


Figure 11. Comparison of the mean hatch rate (± SD) of the clutches derived from females of the three different carotenoids diet groups raised in tap water. Hatch rate is arcsine transformed. See text for statistics.

A.2.4.2 Hatch rate in lake water

In lake water a significant difference in hatch rate of the eggs from the females of the three different carotenoids diet groups could be detected (log likelihood test, Δ *d.f.*=1, χ^2=4.961, *P*=0.026). Post hoc analyses showed that hatch rate of eggs from the females receiving a zero carotenoids diet was significantly higher than the hatch rate of the eggs from the females fed a high carotenoids diet. No significant hatch rate differences were found between eggs of the zero and low carotenoid diet females, or between the eggs of the low and high carotenoid diet females (Tukey post hoc tests, Table 8, Figure 12).

Table 8: Results of the Tukey post hoc tests investigating the differences in mean arcsine transformed hatch rate between females of the different carotenoid diet treatment (*n*=42).

	Δdf	χ^2	*P*
Zero carotenoid diet vs low carotenoid diet	1	2.198	0.138
Zero carotenoid diet vs high carotenoid diet	1	4.941	0.027*
Low carotenoid diet vs high carotenoid diet	1	1.387	0.239

Results reaching the significance level of *P*<0.05 are marked with a *

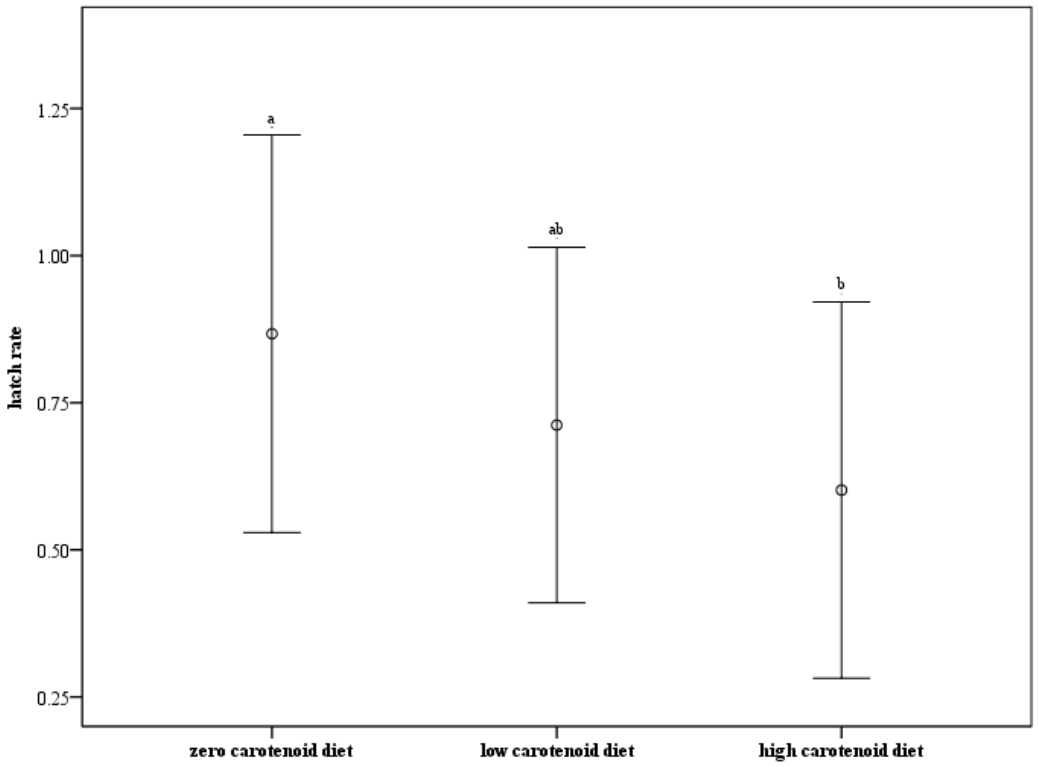


Figure 12. Different mean hatch rate (arcsine transformed, ± SD) of the clutches from the zero, low and high carotenoid diet females raised in lake water. Significant differences can be recognized by the bar annotations. See text for statistics.

A.2.4.3 Female and male random effects on hatch rate

Pooling the data from tap and lake water allowed me to test for female or male random effects on hatch rates combined. Female identity had a significant effect on the hatching success (log likelihood test, Δ *d.f.*=1, χ^2=5.466, *P*=0.019), while the male identity did not have an impact on the percentage of offspring successfully hatching (log likelihood test, Δ *d.f.*=1, χ^2=2.169, *P*=0.1). For further investigations of the parental traits influencing the offspring's hatch rate see A.2.6.

A.2.5 Survival rate during the first week after hatching

A.2.5.1 Survival rate in relation to original clutch size

When survival rate in relation to original clutch size was looked at, the results mirrored the ones found for hatch rate (see *Appendix* 6.3 for detailed statistics). The observed patterns of survival rate of the hatchlings in tap or lake water in

relation to the number of eggs originally present in a clutch were a product of the differential hatch rates in the two raising environments because no significant differences were found in survival when comparing the six different test groups (tap water zero, low and high carotenoid eggs, lake water zero, low and high carotenoid eggs). (Kruskal Wallis, *d.f.*=5, χ^2=4.948, *P*=0.422).

A.2.5.2 Survival rate in relation to hatch rate

When survival rate relative to hatch rate was studied, no significant effect of any of the investigated explanatory variables was found (Table 9). Carotenoid dietary treatment did not have an impact on offspring survival after hatching.

Table 9: Results of the log likelihood tests investigating the variables influencing survival rate during the first week after hatching (*n* = 42).

Explanatory variable	Δ d.f.	χ^2	*P*
Female carotenoid diet × water quality	1	2.655	0.448
Female carotenoid diet	1	0.167	0.683
Water quality	1	1.991	0.158
Clutch size	1	0.949	0.330
Average egg size	1	1.833	0.176

A.2.5.3 Female and male random effects on survival rate in relation to hatch rate

Female identity was found to be significantly influencing the survival of the hatchlings until the age of one week (Log likelihood test, Δ *d.f.*=1, χ^2=8.880, *P*=0.003). However, these results must be considered as an artefact of the impact the few families in which many offspring died (versus many families with no or few dead fish) have on the dataset assuming normal distribution. When investigating the influence of the recorded female traits on survival rates, no significant effects at all were found (see A. 2.6). Male identity did not have an effect on the survival of the hatchlings (Log likelihood test, Δ *d.f.*=1, χ^2=0.909, *P*=0.340).

A.2.6 Female traits influencing the offspring's hatch and survival rate

A.2.6.1 Hatch rate

Of the studied traits, female condition was the only one to influence offspring hatch rate (GLM, Table 10). Females which were in worse condition turned out to have offspring with higher hatch rate (Table 10, Figure 13).

Table 10: Results of the general linar model investigating the female-dependent traits influencing hatch rate (n = 42).

Explanatory variable	*df*	*t*	*P*
Female standard length	4,37	0.298	0.768
Female condition	1,40	-3.687	<0.001*
Clutch size	4,37	-1.167	0.251
Average egg size	4,37	-0.929	0.359

Results reaching the significance level of P<0.05 are marked with a *

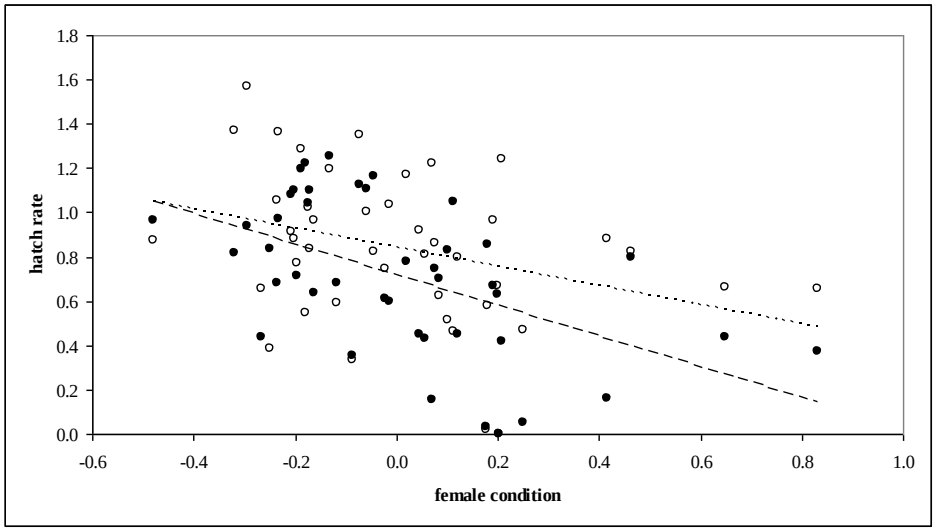


Figure 13. Correlation between female condition and offspring hatch rate in the two different rearing environments (tap water open circles, upper dashed line, lake water black circles, lower dashed line). Hatch rates are arcsine transformed. See text for statistics.

A.2.6.1 Survival rate in relation to hatch rate

None of the investigated female traits was found to influence the survival of the offspring relative to the number of fish which had successfully hatched.

Table 11: Results of the general linear model investigating the female-dependent traits influencing survival rate (n = 42).

Explanatory variable	*df*	*t*	*P*
Female standard length	4,37	0.498	0.621
Female condition	4,37	-0.234	0,816
Clutch size	4,37	-0.368	0.715
Average egg size	4,37	1.057	0.298

Results reaching the significance level of $P<0.05$ are marked with a *

A.2.7 Offspring size six weeks after hatching

There was a significant effect of the interaction between female diet and water quality on offspring size six weeks after hatching (Log likelihood test, Δ *d.f.*=1, χ^2=4.021, P=0.045). Thus, further statistical investigations were done for tap water and lake water separately.

Of the covariates included in the linear mixed model, density of the fish in the raising tanks influenced size at six weeks negatively, while time in the breeding season the eggs had been laid and egg size were found to be influencing the growth rate of the fish positively (Log likelihood tests, Table 12).

Table 12: Results of the log likelihood tests investigating the variables influencing growth rate during the first six weeks after hatching (n = 41).

Explanatory variable	Δ d.f.	χ^2	*P*
Female carotenoid diet × water quality	1	4.021	0.045*
Raising density	1	6.502	0.012*
Date breeding	1	12.542	<0.001*
Average egg size	1	4.149	0.042*

Results reaching the significance level of $P<0.05$ are marked with a *.

A.2.7.1 Offspring size in tap water

A significant difference in the size of the fish at the age of six weeks after hatching could be detected depending on the level of carotenoids their mother had received before egg-laying (log likelihood test, Δ *d.f.*=1, χ^2=4.042, *P*=0.044).

Post hoc tests revealed that the differences in size between offspring from the zero and the high carotenoid diet females only showed a trend to be significant while the differences between the zero and the low carotenoid diet groups as well as the low and high carotenoid diet groups were not significant (Table 13). Offspring from the zero carotenoid diet females showed a trend to be bigger than the offspring from the other two groups.

Table 13: Results of the Tukey post hoc tests investigating the differences in offspring size six weeks after hatching between the offspring of females of the different carotenoid diet treatments (*n*=41).

	Δdf	χ^2	*P*
Zero carotenoid diet vs low carotenoid diet	1	1.331	0.249
Zero carotenoid diet vs high carotenoid diet	1	3.666	0.056
Low carotenoid diet vs high carotenoid diet	1	2.427	0.119

A.2.7.2 Offspring size in lake water

The offspring of females of the three different carotenoid diet groups were not found to be different in size six weeks after hatching (Log likelihood test, Δ *d.f.*=1, χ^2=0.001, *P*=0.971).

A.2.7.3 Female and male random effects on offspring size

Female identity was found to influence offspring size six weeks after hatching (Log likelihood test, Δ *d.f.*=1, χ^2=15.937, *P*<0.001), as was male identity (Log likelihood test, Δ *d.f.*=1, χ^2=4.613, *P*=0.032). See A.2.9. for detailed analyses.

A.2.8 Growth model

To investigate the impact of the raising environment and of carotenoid treatment on offspring growth rate, several growth models were tested using Akaike information criteria (AIC). The model which fit best for the presented data (AIC=1095.807, see *Appendix*) was a two degree polynomial one:

$$size = a + bt + ct^2$$

where *a* is the initial size of the fish, i.e. the size of the hatchlings one week after hatching, *b* is the initial growth rate and describes the growth rate during the early weeks of development, and *c* describe the changes in growth rate during the first six weeks of development. *t* is the time factor.

A. 2.8.1 Effects of female carotenoid diet and water quality on initial growth rate (*b*)

There was a significant effect of the interaction between carotenoid diet treatment and water quality on initial growth rate (Log likelihood test, Table 15). Raising density also influenced initial growth rate although this trend failed statistical significance (Table 14). The other covariates did not have an impact on initial growth rate (Table 14). Average egg size, which was initially included in the covariates was dropped because it did not show a significant effect (P=0.486). Excluding it allowed analysing the whole dataset including the three clutches for which egg data were missing.

Table 14: Results of the log likelihood tests investigating the variables influencing initial growth rate (n = 41).

Explanatory variable	Δ d.f.	χ^2	P
Female carotenoid diet × water quality	1	4.535	0.033*
Raising density	1	3.759	0.053
Date breeding	1	0.016	0.899

Results reaching the significance level of P<0.05 are marked with a *.

Because of the interaction being significant, separate tests for the fish raised in lake water and those reared in tap water were done.

A.2.8.2 Initial growth rate (*b*) in tap water

No difference between offspring of the three carotenoid diet treatment groups could be found in the initial growth rate (Log likelihood test, Δ *d.f.*=1, χ^2=0.250, *P*=0.617, Figure 14).

A.2.8.3 Initial growth rate (*b*) in lake water

The hatchlings derived from females belonging to the different carotenoid diet treatment groups turned out to differ in their initial growth rate (Log likelihood test, Δ *d.f.*=1, χ^2=7.017, *P*=0.008). When the individual diet groups were compared, a significant difference between the growth rates of offspring from the zero versus the high carotenoid diet females could be found (Table 15). Offspring from the zero treatment grew fastest in early life, while initial growth rate was reduced with increasing carotenoids in the mothers' diet, resulting in the offspring of the high carotenoid diet females growing most slowly in the beginning (Figure 14).

Table 15: Results of the Tukey post hoc tests investigating the differences in initial growth rate between the offspring of females of the different carotenoid diet treatments (*n*=41).

	Δdf	χ^2	*P*
Zero carotenoid diet vs low carotenoid diet	1	2.421	0.120
Zero carotenoid diet vs high carotenoid diet	1	7.291	0.007*
Low carotenoid diet vs high carotenoid diet	1	2.317	0.128

Results reaching the significance level of *P*<0.05 are marked with a *

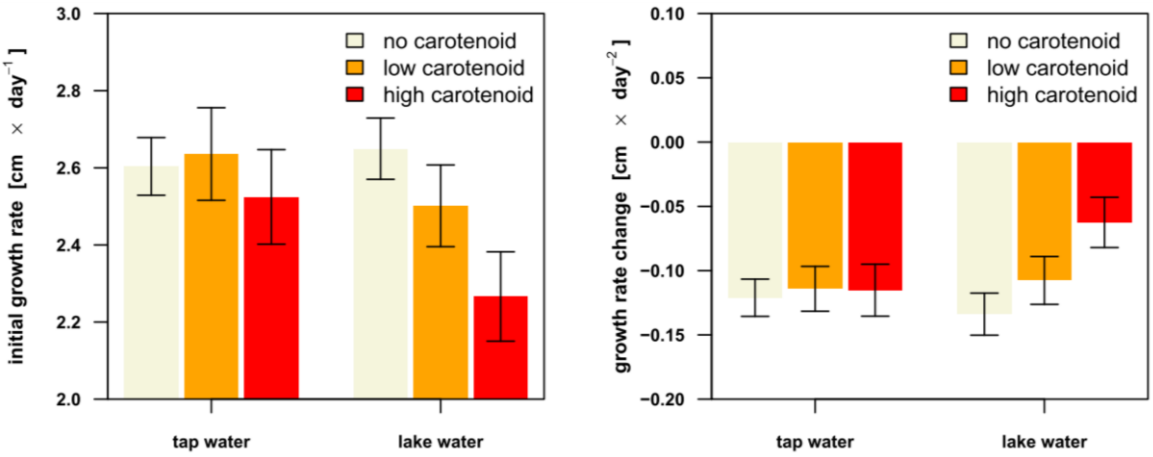


Figure 14. Differences in the initial growth rate (left) and the growth rate change (right) of the offspring from the three different carotenoid diet treatments.

A.2.8.4 Female and male random effects on initial growth rate (*b*)

Female identity significantly influenced offspring initial growth rate (Log likelihood test, Δ *d.f.*=1, χ^2=12.196, *P*<0.001) while no effect of the males could be found (Log likelihood test, Δ *d.f.*=1, χ^2=1.760*10^{-8}, *P*>0.999). See A.2.9. for detailed analyses.

A. 2.8.5 Effects of female carotenoid diet and water quality on growth rate change (*c*)

Again, a significant effect of the interaction of carotenoid diet treatment and water quality was found. As a consequence, separate analyses for the hatchlings raised in tap and lake water were conducted. Both covariates included in the model did not have a significant influence (Log Likelihood tests, Table 16).

Table 16: Results of the log likelihood tests investigating the variables influencing initial growth rate (*n* = 41).

Explanatory variable	Δ d.f.	χ^2	*P*
Female carotenoid diet × water quality	1	6.361	0.012*
Raising density	1	1.244	0.265
Date breeding	1	0,969	0.325

Results reaching the significance level of *P*<0.05 are marked with a *.

A.2.8.6 Growth rate change (*c*) in tap water

No significant difference in growth rate change was found for the offspring of the different carotenoid diet treatment groups (Log likelihood test, Δ *d.f.*=1, χ^2=0.107, *P*=0.743), i.e. growth rate change for offspring of all three dietary treatments was the same over the time of data collection (Figure 14).

A.2.8.7 Growth rate change (c) in lake water

In lake water, hatchlings from the females of the three different diet groups did show a difference in growth rate change (likelihood test, Δ *d.f.*=1, χ^2=8.086, *P*=0.005). Again, the significant differences were found in the comparison between the hatchlings from the zero carotenoid and the high carotenoid diet females (Table 17). Growth rate change was smallest for the zero carotenoid diet group and increased with increasing carotenoid diet (Figure 14).

Table 17: Results of the Tukey post hoc tests investigating the differences in growth rate change between the offspring of females of the different carotenoid diet treatments (*n*=41).

	Δdf	χ^2	*P*
Zero carotenoid diet vs low carotenoid diet	1	2.202	0.138
Zero carotenoid diet vs high carotenoid diet	1	8.750	0.003*
Low carotenoid diet vs high carotenoid diet	1	2.440	0.118

Results reaching the significance level of *P*<0.05 are marked with a *

A.2.8.8 Female and male random effects on growth rate change (c)

Female identity significantly influenced offspring growth rate change (Log likelihood test, Δ *d.f.*=1, χ^2=8.543, *P*=0.004) while no effect of the males could be found (Log likelihood test, Δ *d.f.*=1, χ^2<0.001, *P*>1.000). See A.2.9. for detailed analyses.

A.2.9 Female traits influencing the offspring's size, initial growth rate (*b*) and growth rate change (*c*).

A general linear model was calculated to detect which female variables influenced the size of the offspring at six weeks after hatching. The full model revealed trends of an effect of the SL of the female and of egg size on offspring size (GLM, Table 18).

Table 18: Results of the general linear model investigating the female-dependent variables influencing offspring size six weeks after hatching (n = 41).

Explanatory variable	*df*	*t*	*P*
Female standard length	5,34	1.969	0.063
Female condition	5,34	- 0.485	0.475
Egg coloration	5,34	-0.796	0.420
Clutch size	5,34	0.029	0.775
Average egg size	5,34	1.916	0.068

Results reaching the significance level of $P<0.05$ are marked with a *

When the variables showing a trend of influencing offspring size at the age of 6 weeks were investigated further in the minimal model, only female SL was found to have a significant effect (female SL: $F_{1,39}=7.401$, $t=2.721$, $P=0.010$). The influence was positive, bigger females produced bigger offspring, and there was a positive trend for egg size doing the same (Figures 15 and 16).

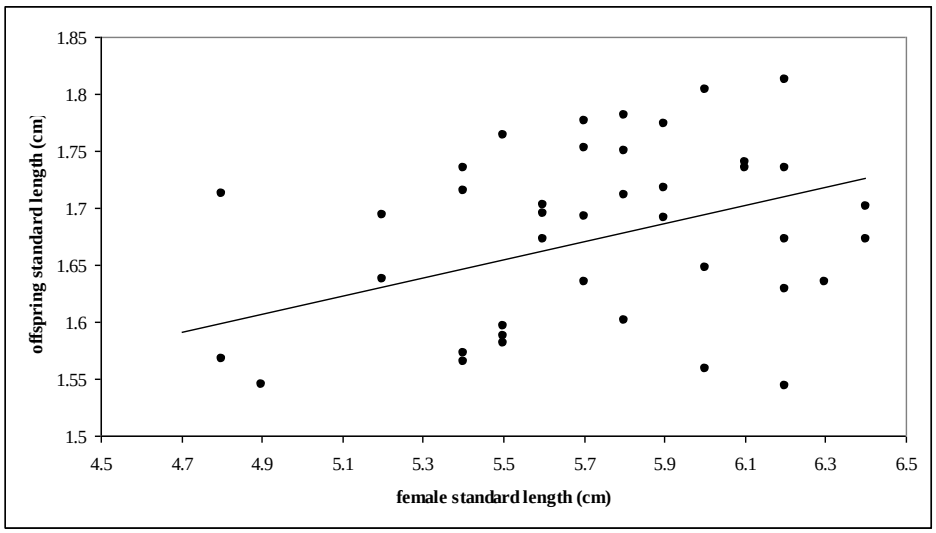


Figure 15. Dependence of offspring size six weeks after hatching on the size of the females. Size was measured as standard length for both generations. *n*=41

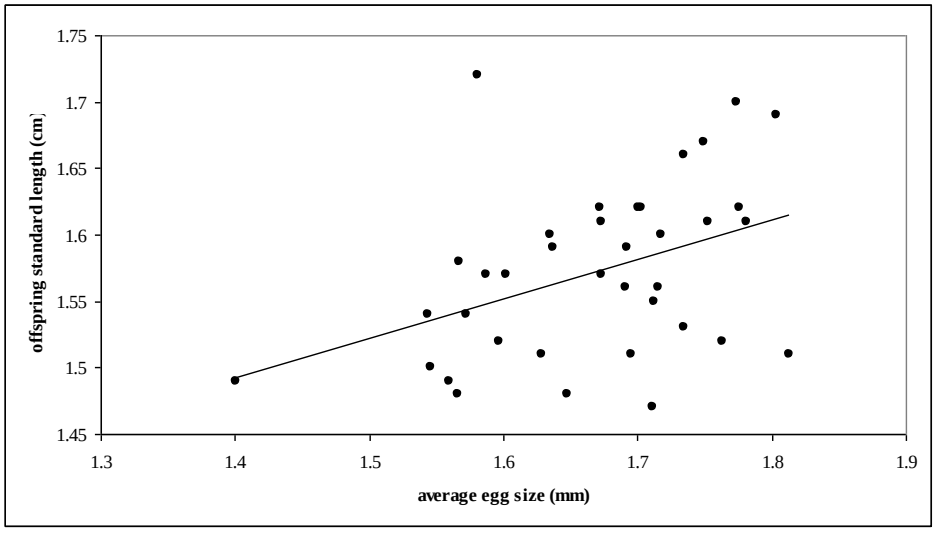


Figure 16. Dependence of offspring size six weeks after hatching on egg size. *n*=41.

When initial growth rate was looked at, it was found that more intensely colored eggs had a lower initial growth rate independent of the dietary treatment the egg-laying females had received ($F_{2,34}$=7,380, t=-3.322, P=0.002). All other investigates variables were not significant (Table 19).

Table 19: Results of the general linear model investigating the female-dependent variables influencing initial growth rate *b* of the hatchlings (*n* = 37).

Explanatory variable	*df*	*t*	*P*
Female standard length	5,31	1.716	0.096
Female condition	5,31	0.541	0.592
Average egg size	5,31	-0.646	0.523
Clutch size	5,31	-0.230	0.820
Egg coloration	2,34	-3.322	0.002*

Results reaching the significance level of $P<0.05$ are marked with a *

In growth rate change *c*, egg coloration also proved to have a significant effect independent of female carotenoid diet, because fish hatched from eggs which were more intensely colored showed a higher change in growth rate ($F_{1,35}$=7,380, t=2.875, P=0.007). The other investigated variables were all found to be non-significant (Table 20).

Table 20: Results of the general linear model investigating the female-dependent variables influencing initial growth rate *b* of the hatchlings (*n* = 37).

Explanatory variable	*df*	*t*	*P*
Female standard length	5,31	-1.655	0.108
Female condition	5,31	0.353	0.726
Average egg size	5,31	1.266	0.215
Clutch size	5,31	0.491	0.627

Results reaching the significance level of $P<0.05$ are marked with a *

A.3 Discussion

Summarizing the main findings, this study indicates that there is a connection between the level of carotenoids in the diet of female sticklebacks and the chromaticity of the eggs they produce. Egg chromaticity mirrors egg carotenoid content to certain extends. Carotenoids in the egg have an impact on offspring fitness under certain circumstances. Females receiving artificial food with no or a low amount of carotenoids produced eggs which did not significantly differ from each other in chromaticity and were similarly colored to eggs produced by females fed a natural food source. Contrasting, females supplemented with food containing high amounts of carotenoids laid eggs which were more than twice as intensely colored (mean egg chromaticity) as the eggs from the other groups. Egg size and egg number were not affected by the amount of carotenoids the egg laying female received in its diet. When fitness parameters of the offspring of the three carotenoid diet group females were compared, I found that raising the fish in sterile lab conditions (tap water) yielded different results than rearing them in a more natural environment (lake water). When eggs/hatchlings were raised in tap water, no differences were found between female diet groups in terms of hatching success, initial growth rate and growth rate change of the offspring. There was a trend of hatchling size six weeks after hatching to be significantly lower for the higher supplemented diet group in two out of the three treatment-comparisons (zero vs. high and low vs. high carotenoid diet). For the offspring raised in lake water, hatch rate and initial growth rate of the offspring declined as the amount of carotenoids the mothers had received rose. Growth rate change was highest for offspring of the high carotenoid diet females and declined as carotenoid amount in the food of the mother declined. In other words, the offspring with a low initial growth rate grew faster in the later weeks of life, while the fish with a high initial growth rate later grew more slowly. This resulted in no significant size differences between offspring of the carotenoid diet group females at the age of six weeks after hatching. For both tap and lake water raised fish, survival rate after hatching merely reflected hatch rate, but was not affected by carotenoid diet treatment of the mothers. I found that hatch rate of the offspring declined as female condition rose. The influence of female

standard length on offspring size was still recognizable six weeks after hatching, with females with a higher standard length producing bigger offspring.

The amounts of dietary carotenoids chosen in this experiment were well within the range usually used when effects of carotenoid supplementation are studied in fish. This range lies between 0 and 200 mg of carotenoids per kg feed (e.g. (Bjerkeng *et al.*, 1990; Christiansen & Torrissen, 1997; Verakunpiriya *et al.*, 1997; Choubert, 1998; Grether, 2000; Amar *et al.*, 2004; Ahmadi *et al.*, 2006; Sawanboonchun *et al.*, 2008; Garner *et al.*, 2010; Lakeh *et al.*, 2010; Pike *et al.*, 2010). In nature, sticklebacks are known to feed on a carotenoid-rich diet mainly consisting of small Crustaceans, aquatic insects and larvae, worms of various kinds, drowned aerial insects and the eggs and fry of fish including their own. However, there are differences in diet between populations, different sized fish and seasons (Hynes, 1950). Various kinds of carotenoids, including astaxanthin and lutein, have been repeatedly identified in the skin of male sticklebacks in breeding coloration (i.e. Wedekind *et al.*, 1998; Pike *et al.*, 2007a). When males were fed artificial diets of either low (20µg/kg feed) or high (200µg/kg feed) levels of total carotenoids, skin analyses showed that the amount of carotenoids in the nuptial coloration of these artificially carotenoid-supplemented fish were within the range found in the skin of freshly-caught wild breeding males from the same population (Wedekind *et al.*, 1998; Pike *et al.*, 2007a). Thus the amount of carotenoids chosen for artificial supplementation of the fish was corresponding to the amount of carotenoids wild males encountered in their diet. It was shown in the same study that females respond to dietary carotenoid supplementation similarly as males in their body carotenoid levels, with the females receiving the high carotenoid diet exhibiting higher amounts of body carotenoids than females of the low carotenoid diet. The levels of total carotenoids used by Pike et al. (2007) were similar (for the low level) or the same (for the high level) to the ones I used. Therefore carotenoid supplementation did not fall below or exceed the levels of carotenoids present in a wild stickleback's diet. This means it is unlikely, that the negative effects of carotenoids found for the fish raised in lake water were a consequence of artificially high carotenoid concentrations.

Considering my results the impact of carotenoid level in the diet of the female on offspring traits and fitness can be looked at from two perspectives:

(1) Carotenoids and egg chromaticity

I showed that egg coloration in sticklebacks can be caused by the carotenoids astaxanthin and/or lutein or some kind of carotenoid bioconverted from them. It appears that under a certain threshold of carotenoid content of the diet, egg chromaticity is similar, but starts to rise when the threshold level is passed. My results suggest that egg chromaticity might only be a good measure of carotenoid content in the high carotenoid treatment: when fitness parameters were investigated in lake water, the results from the fish supplemented a low carotenoid amount were always intermediate to the ones from the fish supplemented with zero and high carotenoid levels, although egg color of the females from the zero and the low carotenoid diet was highly comparable.

I supplemented the females with equal amounts of astaxanthin and lutein each in the low and high carotenoid diet treatment. Due to my experimental design, it can not be said whether astaxanthin or lutein alone caused egg coloration, or if it was a combination of those two carotenoids. If the first case was true, the threshold above which egg chromaticity starts to rise would lie somewhere between 20 and 100mg of astaxanthin or lutein per kg feed. In the latter case, it would be found between 40 and 200mg of total carotenoids per kg feed. At the simplest level, chemical analyses on the types of carotenoids present in the eggs in combination with experiments using only one kind of carotenoid for dietary supplementation could answer the question whether only one or more than one dietary carotenoid is responsible for egg chromaticity in the stickleback.

I compared females fed an artificial carotenoid enriched diet (*Part A,* Vienna experiment) with females fed chironomid larvae (*Part B*, Bonn experiment) and found that mean egg coloration from females fed no or low amounts of the artificial diet is similar to egg coloration from females fed a natural diet. The red color of chironomid larvae, on which Bonn fish were fed, is not due to carotenoids but haemoglobin in their extracellular substance (Walshe, 1947; Czeczuga, 1961; Cabrerizo Ballesteros *et al.*, 2006). However, the larvae are

known to contain carotenoids as well (Czeczuga, 1961). Since wild sticklebacks usually feed on a variety of different species (Hynes, 1950) which supposedly contain different levels of carotenoids, it is likely that the range of egg chromaticity found in my study – from rather pale to very intensely yellowish colored eggs - is also present in eggs of wild females. To prove this however, it would be necessary to catch ripe wild females, breed them immediately after catching and assess the variation in egg coloration with methods similar to the ones I used. This has unfortunately never been done in any stickleback population.

Egg coloration did not rise continuously from the zero to the low to the high dietary carotenoid content in the studied population. In three-spined sticklebacks, data on the influence of carotenoids on egg coloration and carotenoid content of eggs have been lacking so far. Some information on the conversion rate from dietary carotenoids into egg carotenoids can be drawn from studies investigating other fish species, even if the focus of these studies has not been egg coloration as such. Both in Atlantic cod and in rainbow trout it has been shown that enhancing the level of astaxanthin supplementation in the mother generally also enhances the carotenoid content of her eggs (Ahmadi *et al.*, 2006; Sawanboonchun *et al.*, 2008). In the rainbow trout, females receiving diets containing either 0.1, 13, 33, 65 or 93 mg astaxanthin/kg feed (numbers rounded) all incorporated astaxanthin into their eggs. However, while raising the dietary astaxanthin content from 0.1 to 33 mg/kg feed resulted in a rise of egg astaxanthin content from 2 to 22 mg/kg. Elevating the dietary astaxanthin level further from 33 to 65 to 93 mg/kg feed led to an egg astaxanthin content rising only from 22 to 25 to 30 mg/kg. Thus, raising the dietary astaxanthin content above a certain level did not result in relevant increase of astaxanthin level in the eggs any more (Ahmadi *et al.*, 2006).

To my knowledge, there are only two other studies in fish in which carotenoid content of maternal diet and its' connection to egg coloration has been investigated. In the rainbow trout, egg coloration rose as dietary supplementation of the females with canthaxanthin rose. However, the aim of the study in rainbow trout was to evaluate possible enhancement female fecundity by carotenoid supplementation and thus the author did not focus on

egg color as such (Harris, 1984). In the yellowtail, reproductive success measured as number of eggs produced, fertilization rate, egg and oil globule diameter, hatch rate and rate of healthy larvae rose as dietary supplementation level was increased from 0 to 20 to 30 mg of astaxanthin per kg feed, while it sank when further raising the carotenoid content to 40 mg per kg feed (Verakunpiriya *et al.*, 1997). Egg coloration responded in the same manner as did reproductive success, thus the eggs from the females fed no carotenoids were only pale yellow, became more and more yellow in the 20 and 30mg/kg diet group, but sank again in the 40mg/kg diet group. It was concluded that these results point out the limited ability of yellowtail fish to use bioconversion of astaxanthin into zeaxanthin, which is known to be the main carotenoid component in yellowtail eggs (Miki *et al.*, 1982). Thus eggs from the highest supplementation group did actually contain less carotenoid as judged from coloration as did the 30mg/kg supplementation group, in which reproductive success was highest. It can not be ruled out from these data, that if the fish had been able to also convert the 40mg carotenoid dose, reproductive success would have been even further enhanced. The results in the yellowtail are therefore not comparable to the ones I found in sticklebacks, where reproductive success was lowered as carotenoid content as judged from egg coloration rose. Thus, it seems that effects of carotenoids in the diet on the coloration of eggs can be quite species-specific. Not all carotenoids or doses of carotenoids used in the diet might be accessible for fish to convert into egg carotenoids, which makes the chemically based assessment of egg carotenoids and the knowledge about possible utilization and bioconversion of the predominantly used dietary carotenoids essential.

(2) Offspring fitness differences caused by level of maternal carotenoid diet:
My results show negative effects of carotenoids on both hatching success and growth rate, but only in lake water. Thus they point out the major importance of the environment in which the effects of carotenoids are tested. Coloration of eggs from females fed the zero and the low carotenoid level diet were not significantly different from each other, but effects on offspring fitness differed between these groups in lake water – the strength of effects in the low

carotenoid level group usually being placed between the zero and the high carotenoid level group.

My results came unexpected, as carotenoid investment of females into their offspring has repeatedly been described as especially positive in early life stages, when cell activity and oxidative metabolism are high (Møller *et al.*, 2000). I investigated exactly these stages of very fast development, from egg laying to hatching up until the first few weeks after hatching. Some studies in wild birds suggest that carotenoid concentration in eggs may on average be lower than the concentration that might be most beneficial for the offspring. Experimental manipulation of egg carotenoid content is usually done within the range of reported natural variation (e.g. Saino et al. 2011). In contrast to the results I present here, possible costs of carotenoid content of the egg have hardly been investigated or found in birds. It is mainly positive or a lack of effects that are reported (reviewed in (Møller *et al.*, 2000). My study suggests that the reason for this might be that (1) the carotenoid level – although probably within natural variation - at which negative effects would have occurred was not reached or (2) the maintenance conditions of animals prohibited finding effects that would have been present in the wild. Additionally, there is of course the possibility that carotenoid effects are taxon or even species specific and a general conclusion cannot be drawn.

The higher the level of maternal carotenoid diet was, the lower the hatch rate was in lake water, while there was no effect on hatch rate in tap water. In numbers, mean hatch rate of the eggs from the zero carotenoid diet treatment females in lake water was comparable to the hatch rates in tap water, while hatch rates from eggs of the low and high carotenoid diet groups in lake water were smaller. Thus, it cannot be concluded that water quality as such had a general negative effect on hatch rate, as this would have lowered hatch rate in all dietary treatments in lake water compared to tap water. The survival of embryos in eggs can be harmed by a variety of pathogens and parasites, the likelihood of which can be reduced by actions of the parental generation, which make embryos better equipped to withstand such challenges. This can for example happen via deposition of maternal antibodies into the eggs shortly before egg laying (e.g. (Smith *et al.*, 1994; Swain *et al.*, 2006), but also by females choosing a mating

partner with which they can produce offspring high in MHC diversity, which has been shown in sticklebacks (Kurtz *et al.*, 2004). Paternal investment also plays a role in enhancing the offspring's resistance against pathogens in sticklebacks, as the males' glue which is used to bind the vegetation in the nest was found to have antimicrobial properties which decrease the growth rate of bacteria and fungi and thus enhance maturation and hatch rate (Little *et al.*, 2008). It seems unlikely that in my study, exposure of the eggs to pathogens was the same in clean tap water (drinking quality) as it was in lake water. Thus, the conclusion can be drawn that in lake water, higher carotenoid content of the egg by some mechanism lowered the embryos resistibility to pathogens present in the surrounding medium. In tap water, the supposedly relatively low concentration of pathogens prevented that eggs with rising carotenoid content were harmed in the same rates as they were in lake water. If carotenoids themselves had the power to lower survival of the embryos and manifestation of bacteria and fungi on the eggs happened only as a consequence of embryo death caused by the carotenoids, the eggs of the zero carotenoid diet females in lake water would have had a lower mean hatch rate as the eggs in tap water, which was not the case. This clearly points out the importance of the interaction between carotenoids and environment. Since it seems unlikely that in the wild, conditions are as sterile as they are in tap water, the effects of carotenoids on hatch rate in lake water are believed to be more representative for the situation in wild sticklebacks.

Several studies on other fish species failed to find any carotenoid-mediated benefits on offspring fitness although methods and parameters investigated were similar to my study (Christiansen & Torrissen, 1997; Choubert, 1998; Amar *et al.*, 2000; Grether *et al.*, 2008). In birds, carotenoid provision as maternal effect has been investigated in the wild and/or natural stressors like ecto- or endoparasites were used to challenge the embryos/hatchlings immune system (e.g. Berthouly *et al.*, 2008; Ewen *et al.*, 2009). These studies might produce more reliable results because they mimic more natural conditions. In fish, however, the reason for a lack of significant results on carotenoid-mediated effects might not necessarily be that there really are no effects, but that they were simply not detected under the particular conditions the fish were kept in in the mentioned studies.

Negative effects of dietary carotenoids are reported in literature very rarely. However, it was found that in humans, β-carotene can have a negative effect on the incidence of lung cancer and cardiovascular disease although β-carotene and a variety of other carotenoids have long been praised for their positive effects in the treatment of chronic diseases in humans (The Alpha-tocopherol Beta Carotene Cancer Prevention Study, 1994; Mayne, 1996; Omenn *et al.*, 1996). This points out that the same type of carotenoid can have conflicting effects.

In tap water, offspring from all dietary treatment groups exhibited comparable initial growth rates and growth rate change. At the age of six weeks after hatching, the offspring from the zero carotenoid diet group showed a trend to be bigger than offspring from the high carotenoid diet females. However, results were only marginally significant and larger studies are needed to clarify if there really is a carotenoid-dependent effect on offspring size in the tap water treatment.

More interestingly, initial growth rate of the fish raised in lake water was reduced in comparison to the tap water fish. Yet, while hatchlings from the high carotenoid diet mothers grew more slowly than the other two groups shortly after hatching, they started developing more rapidly in the later weeks. As a result of these incidents, fish from all carotenoid diet groups were similar in size at the age of six weeks. This might be a hint for egg contents playing an important – in this case significantly negative - role in early development, while factors like the environment the hatchlings are raised in or diet of the juvenile fish themselves get more and more important in later life. The absorption of the yolk sac by alevins prolongs into the time period after hatching (e.g. (Alanara, 1993; Zhang *et al.*, 1995). Maternal effects mediated by the contents of the yolk sac can be present up until it is completely depleted. In the stickleback complete yolk absorption is reached four days after hatching (Swarup, 1958). It seems likely that the negative effects of high amounts of carotenoids deposited into the eggs by the females belonging to the high carotenoid diet group hindered initial growth rate of their offspring up until the young fish had absorbed their yolk sac and were completely dependent on their own energetics. It could take some time until the disadvantages related to higher carotenoid content of the egg yolk experienced during yolk sac absorption can be compensated by the juvenile fish.

Thus, the ongoing differences between offspring from the different diet groups at older life stages could be a result of different influences of maternal effects in the early life stages.

Since alevins and fry in both tap and lake water were fed the same food, it can be asked why initial growth rate and growth rate change was not affected by dietary treatment of the mother in tap water, but this was the case in lake water. It might be possible that in lake water, after maternal effects of yolk sac contents become insignificant, there are more nutrients which fry can somehow benefit from in their development, so that they are able to make up for possible disadvantages by maternal effects in early life, which could not be the case in the sterile tap water. This would explain why in lake water, the initially slow growing hatchlings from the high carotenoid diet mothers are able to make up in size to the offspring from the other, initially faster growing conspecifics as soon as they escape from the "regime" of the in this case negative effect of maternal carotenoid deposition. Algae in the water might have played a role. Even though sticklebacks do not actively feed on them, there is the possibility of passive intake of algae when swallowing food. It was found in guppies that algal intake enhanced growth, which might be the result of non-digestible carbohydrates within algae having an important function in physiological systems (Karino & Haijima, 2004).

While hatch rate was diminished in the offspring of females incorporating a lot of carotenoids into their eggs, the successfully hatched individuals did not suffer from disadvantages in terms of survival under laboratory conditions. However, for wild fish, predation on fry must be taken into account. Although the general validity of the "bigger is better" hypothesis has been questioned (Litvak & Leggett, 1992), it is generally believed that it is beneficial for juvenile fish to quickly reach a threshold length above which susceptibility to predators decreases more quickly than encounter rate increases (Cowan *et al.*, 1996) or they outgrow some predator species thus narrowing the predator spectrum which they are suitable prey for (Taylor, 2003). Additionally, in juvenile fish in moderate climate regions it has been shown that larger juvenile conspecifics are better able to withstand physical extremes and endure longer periods without food (reviewed in Sogard, 1997). Since hatchlings from females incorporating a

lot of carotenoids into their eggs had a lower growth rate shortly after hatching, they would have possibly encountered reduced levels of survival compared to the larger progeny from females investing less carotenoid into their offspring.

With carotenoids having all the negative effects reported here for conditions in lake water, it seems unlikely that females would actively incorporate high amounts of carotenoids into their eggs and thus harm their own inclusive fitness. From this it can only be concluded that at least above a certain dietary carotenoid amount, females passively pass on level of carotenoid concentration to their eggs. Alternatively, it could be the case that even though carotenoids have a negative effect on hatch rate and growth, some kind of positive effect on offspring fitness parameters not tested in this study outweighs the negative effects. High amounts of carotenoids in eggs could then still be beneficial for the individual offspring.

Assessment of offspring value

Another quite counterintuitive result of my study was that females in worse condition produced eggs with a higher mean hatch rate. One explanation for this might be the following: Females in worse condition made the decision to invest a lot into their current offspring, not knowing whether they would be able to reproduce another time. To maximize lifetime reproductive success, females should consider the value of the current offspring in relation to future reproductive possibilities and base their decision on how much to invest into the current offspring on this value. For stickleback males it has for example been shown that later in the breeding season, the effect of the presence of predators on conspicuousness of the males breeding coloration weakens, since future reproductive events become more and more unlikely and the males are willing to take more risks (Candolin, 1998). Since female sticklebacks do not engage in parental care, they can only maximise their reproductive success for a given batch of eggs by influencing egg properties, for example by transferring nutrients and other beneficial components into the eggs which enhance the offspring's fitness. It seems that this was exactly what the females in bad condition did in my study. As a consequence they managed to enhance the current offspring's' fitness by enhancing hatching success. Additionally, since

females were kept in tanks where they did not have any visual contact to males, they might have assessed mating opportunities as very low, which could have further enhanced their investment into current reproduction, especially in the females which were in worst condition. A previous study found lowered food ration to influence mainly inter-spawning interval in stickleback females and did not change egg characteristics like size or lipid content (Ali & Wootton, 1999). I however showed that low body condition might have an impact on individual clutch fecundity via egg components. However, there is an alternative explanation for the negative correlation between female condition and offspring hatch rate. Since I used weight after spawning to calculate the condition of the females, it could be the case that females in good condition could actually invest the most into their offspring, thus producing eggs with a high hatch rate. It can be imagined that as a consequence of this investment, females who were in good condition before spawning would suffer a cost resulting in them having a dramatically lowered condition after spawning. More investigations on this matter would be necessary to clarify which explanation is more likely to explain the negative correlation between female condition and clutch size.

Eggs from big clutches showed smaller average egg size, and different egg size led to body length differences still recognizable in the juvenile fish at the age of 6 weeks after hatching. For reasons already discussed above, hatching from a bigger egg might be beneficial for the offspring. Bigger females and females in better condition factor laid more eggs. Additionally, bigger females on average produced clutches with bigger eggs and a trend of the same effect was found for females with higher condition factor. Combining these data it is shown that a bigger female will generally be able to produce more eggs of a given size than a smaller female. For a stickleback male, having a limit on how many eggs it can care for at a time, it would thus be beneficial to prefer the bigger female when given the choice, since it would gain more eggs more quickly and save costs of courtship. My data corroborate previous findings in the stickleback, which state that bigger females lay heavier and more eggs, and it has been shown that attractive males do indeed show a preference for those females (Kraak & Bakker, 1998). While Kraak & Bakker only hypothesise about bigger eggs growing faster and thus possibly facing the benefit of outgrowing certain predators more quickly, my data indeed showed that egg size differences

result in size differences in juvenile fish. While growth rate in the course of the first post-hatching weeks of life as such was not investigated in context with egg size in my study, the size differences at the age of six weeks which correspond to egg size differences can still be interpreted as a result of hatchlings from bigger eggs reaching a certain size more quickly than hatchlings from smaller eggs.

It has never been investigated in the species whether the usually reported differences between clutches in egg color result in differential paternal investment. With the negative fitness consequences of more yellowishly colored eggs reported here, it might be plausible that males would prefer to invest more into less intensely colored eggs. Firstly, they could do so by preferentially mating with females that lay less yellowishly colored eggs. However, this would imply that there are female traits by which they can assess the coloration of the eggs. Two-spotted gobies have been shown to advertise the carotenoid content of their gonads by their belly coloration, which is caused by the skin on the belly being transparent, so that gonad coloration responding to more or less carotenoid content is directly visible through the skin (Svensson *et al.*, 2005; Svensson *et al.*, 2009b) Stickleback females of the studied population however do not possess transparent skin which could make carotenoid content of the eggs directly visible, nor do they show any suspicious coloration that could be an indirect hint for carotenoid investment abilities. If any, other traits on the females' body than coloration would have to be present by which the males would assess the quality of their mates in terms of carotenoid investment into the eggs. Secondly, males could adjust their brood caring behaviour according to egg coloration and invest more in paternal care if the eggs in the nest are less intensely colored. Furthermore, male sticklebacks are known to engage in filial cannibalism, which is generally common in teleost fish (reviewed in (Manica, 2002). There are several alternative hypotheses explaining how the evolution of filial cannibalism could be enhanced, one of them being that parents could selectively cannibalize lower quality offspring by partial filial cannibalism (Klug & Bonsall, 2007). Stickleback males have been found to be able to make fine-tuned decisions in their brood caring behavior, for example being able to distinguish foreign eggs (which were fertilized by another male) from their own by egg cues alone (Frommen *et al.*, 2007). Males were more likely to

cannibalize all eggs in the nest when percentage of foreign eggs was high (Mehlis *et al.*, 2010). They are also known to remove dead eggs from the nest (Wootton, 1984). Thus it might be possible that they would be able to distinguish between differently colored egg batches in the nest and be more likely to engage in total filial cannibalism if average egg chromaticity is high, or even be capable of partial filial cannibalism selectively removing more intensely colored eggs. Further studies that investigate possible connections between effort of paternal care and/or filial cannibalism in dependence of clutch coloration are necessary to answer the question if males preferentially care for clutches less intensely colored, which have a supposedly higher fitness in terms of hatching success and growth.

Conclusions

Although carotenoids are usually believed to have various positive effects, I found that they can reduce viability of stickleback offspring. Conditions under which effects of carotenoids are studied proved to be of major importance in my investigations. On the background of these findings, previous studies should be re-evaluated, especially the ones reporting a lack of effects of carotenoids on the studied organism.

Since carotenoids had an impact on offspring fitness and carotenoid supplementation of the females led to a difference in egg coloration between diet groups, there is the potential of egg coloration to be an ornament by which parents could assess offspring quality and make decisions about parental investment. The three-spined stickleback seems to be a species perfect for further investigating the possible importance of egg color for parental care.

Part B: Sexually antagonistic genes in carotenoid investment?

B.1 Material and Method

B.1.1 Study population

Adult three-spined sticklebacks were caught during their spring migration in the year 2008 on the Island of Texel, the Netherlands (for detailed information about the population see A.1.1). After capturing, fish were transported to the Institute for Evolutionary Biology and Ecology of Bonn University, Germany. Until being used for the experiment, males and females were housed together in 750 l outside tanks which had air ventilation and a constant supply of tap water (flow rate 3 l/min). Fish were fed once a day with frozen bloodworms *Chironomus* spp. in excess.

B.1.2 Experimental design

B.1.2.1 Parental generation

B.1.2.1.1 Breeding

Individual males showing nuptial colouration were transferred into 12 liter tanks (30x20x20cm). Visual contact was prevented by placing opaque partitions between the tanks. Each tank was equipped with 2g of Java moss (*Vesicularia dubyana*) as nesting material and a sand-filled petri dish (Ø9cm) placed at the back of the tank as nest side. Water had a temperature of 17 ± 1 °C and was aerated by an air stone. Lightning conditions were set to a 16:8 light:dark cycle with one hour of twilight. Illumination came from natural true spectrum lighting (Co. True-Light: Natural Daylight 5500) emitting light in the visible (400-700nm) and the UV (320-400nm) range. Temperature in the breeding room was held at 17±1°C. Males were fed once a day with frozen bloodworms *Chironomus* spec. Nest building was stimulated by presenting a receptive female for 15 minutes every day. Males built their nests within 24 and 72 hours after

being placed into the breeding tanks (Mehlis, personal communication) and were used for breeding on the 4th day after having finished nest-building. Each male was used only once.

For breeding, females were randomly assigned to the males. (For determination of sexes see A.1.2.1.1). In total, 29 breeding pairs were used (29 families produced). The breeding of the fish was conducted as described in A.1.2.1.2. Standard length of males and females was measured before being placed into the breeding tank. Additionally, the weight of the males was measured directly before they were being transferred from the holding to the breeding tanks and again two hours after spawning. Weight of the females was recorded directly before and after egg-laying. The condition of the males and the females was calculated from the length and weight measurements as the residual of the linear regression.

B.1.2.1.2 Measuring male coloration

Color measurement followed the description in (Rick & Bakker, 2008). Two hours after spawning, males were removed from the tanks and male chromaticity was measured using an Avantes AVS-USB2000 spectrophotometer. For determining chromaticity, males were quickly decapitated to prohibit measurement errors caused by body movements and placed on their side (with the left lateral side facing upwards) onto a piece of black fabric. Measurements were taken on the orange to red cheek region directly below the eye. A bifurcated, 200-µm fiber optic probe providing unidirectional illumination and recording was placed in a 90° angle onto the fish's' body surface. The probe measured reflectance intensity relative to a 98% white reflectance standard (Spectralon WS-2) over the range of 300-700nm. Resolution in wavelength was about 0.5nm. An Avantes DH-2000 deuterium-halogen light source (200-1100nm) was used for illumination. To avoid direct contact between the probe and the fish's' body and to standardize the distance between sensor and the fish's surface, the end of the probe was inserted into a pipette tip which allowed taking standardized measurements from a 3mm distance. By darkening the pipette tip with a black marker, it was ensured that no ambient light fell on the sampled body region. Data were recorded with Spectrawin 5.1 (Avantes) and imported into Microsoft Excel. Chromaticity on the cheek was measured 15 times in succession without moving the probe and an average value for every

male was calculated from these data. Measurements were finished within around one minute, thus a possible color change after the decapitation of the males was minimized (Rick & Bakker, 2008).

B.1.2.2 F1 Offspring

B.1.2.2.1 Maintenance until adulthood

Clutches were taken from the males' nest and the offspring was raised in family groups. The hatchlings were initially housed in 1 liter tanks equipped with an air stone. Water was changed once a day. At the age of three weeks they were transferred into 30x20x20 cm tanks aerated by an air stone at a maximum density of 50 fish per tank. From the age of 3 month until death they were housed in 50x30x30 cm tanks in which water was filtered and aerated by a Hobby gully filter. A third of the water volume was replenished by tab water every week. During the first month after hatching the F1 generation was fed with live *Artemia* nauplii and afterwards the diet was switched to frozen bloodworms *Chironomus* spp. The same light source as described for the parental generation was used. From hatching in summer 2008 until the 1st October 2008 light conditions were set to a 16:8 light:dark cycle including one hour of twilight. From October 2008 to the 13th of May 2009 the fish were kept on winter conditions with a 8:16 light:dark cycle. Then the light was again switched to summer (i.e. breeding) conditions to stimulate the reproduction of the meanwhile adult F1 generation (Borg *et al.*, 2004). During the whole period room temperature was kept at 17±1°C (Frommen *et al.*, 2008).

B.1.2.2.2 Measuring egg characteristics of F1 females

After having reached sexual maturity, a gravid female of each of the 29 families was striped and the eggs were counted and artificially fertilized with sperm originating from a randomly chosen male (see Bakker, Zbinden et al 2006 for details). Two hours after fertilization a sample of 9 eggs per female was photographed under the microscope using a HV-C20AMP Camera (Hitachi Denshi Ltd.) which was controlled by a computer using the Diskus 4.6 program. Before being photographed, eggs were placed for a few seconds on a piece of dry paper to remove adherent water from the surface. Then the eggs were transferred onto a microscope slide and placed under the microscope. A black piece of fabric was placed under the microscope slide and a piece of mm-paper

as well as a standardized white Munsell card (9.5) was visible on each picture (Bakker & Mundwiler, 1994). Before taking the first picture, the white balance was adjusted (3200 K default setting, brightness 19db, contrast 0.45). Illumination of the eggs came from a cold light (KL 1500, intensity 4, focal aperture c). Two light bulbs were placed in a 9cm distance from the microscope slide and with 1 ½ cm distance from the loupe of the microscope. Measurements of (average) egg coloration and calculations of (average) egg size were done exactly as described in A.1.2.2.1. Pictures of the eggs were taken between the 29th of June to the 23rd of July 2009.

B.1.3 Statistical Analyses

General linear models (GLMs) were calculated with (1) egg coloration, (2) egg size and (3) clutch size as the dependent variable. Standard length and condition of the parental generation (males and females), male cheek chromaticity in the parental generation, and standard length and condition of the F1 females (egg-laying) were used as explanatory variables in all models. Depending on the dependent variable investigated, egg coloration, egg size and clutch size were added as explanatory variables as well. Only main effects were considered.

For two males, data on cheek chromaticity could not be reported. Thus, sample size was reduced to n=27 for all chromaticity analyses. Additionally, standard length and weight data were lost for one breeding pair in the parental generation due to technical reasons. Thus, sample size is reduced in the cases where these data were included in the statistical models.

After calculating the full model, data were reduced to the corresponding minimal or null model by excluding the non-significant variables one by one.

B.2 Results

B.2.1 Egg coloration

The chromaticity of the egg-laying female's father had no significant effect on the clutch's average egg coloration (GLM, $F_{5,21}$=2.256, t=-1.347, P=0.192, Table 21). Of the chosen covariates, only clutch size was significantly related to egg chromaticity with bigger clutches being more intensely colored (GLM, $F_{5,21}$=2.441, t=2.441, P=0.024, Table 22, Figure 17). None of the other investigated covariates influenced egg coloration (GLM, all P>0.228, Table 21).

Table 21: Results of the GLM investigating the variables influencing egg coloration. n=27.

Explanatory variable	*df*	*t*	*P*
Cheek chromaticity of female's father	5,21	-1.347	0.192
Female standard length	5,21	-0.266	0.793
Female condition	5,21	0.486	0.632
Clutch size	5,21	2.441	0.024*
Average egg size	5,21	-1.243	0.228

Results reaching the significance level of P<0.05 are marked with a *.

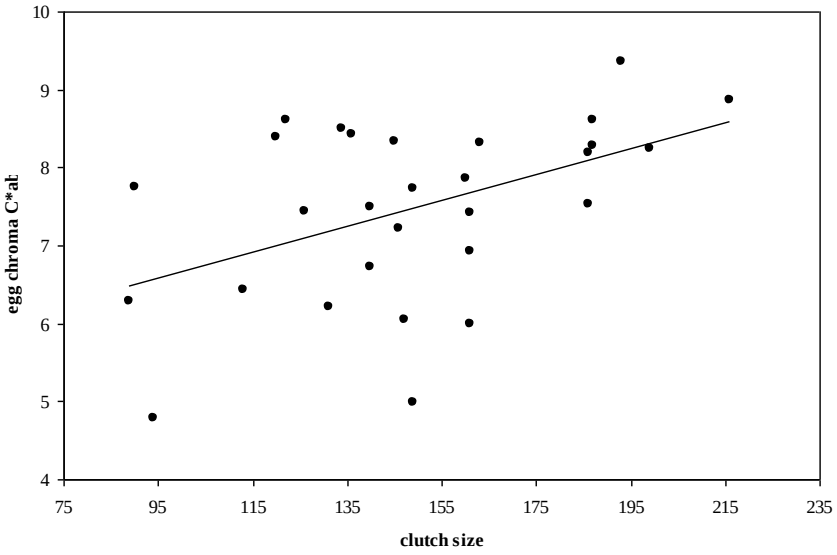


Figure 17. Egg coloration measured as chroma (C^*_{ab}) was more intense in clutches with many eggs.

Reducing the data to the minimal model did not change the significance of any of the variables in the full model and clutch size was the only covariate found to be influencing egg coloration ($F_{1,27}$=7.697, t=-2.774, P=0.001).

B.2.2 Clutch size

Egg color was the only variable found to be significantly influencing clutch size. Bigger clutches contained eggs that were on average more intensely colored (Table 22). This influence of coloration on clutch size was already shown in B.2.1, Figure 17.

Table 22: Results of the GLM investigating the variables influencing clutch size. *n*=27.

Explanatory variable	*df*	*t*	*P*
Cheek chromaticity of female's father	5,21	0.246	0.808
Female standard length	5,21	1.915	0.069
Female condition	5,21	-1.154	0.262
Egg coloration	5,21	2.441	0.024*
Average egg size	5,21	-0.320	0.752

Results reaching the significance level of P<0.05 are marked with a *.

Consistent with the full model, the minimal model found only egg coloration to be significantly correlated with clutch size ($F_{1,27}$=7.697, *t*=-2.774, *P*=0.010).

B.2.3 Egg size

The same covariates as mentioned in B.2.1 were used in the GLM investigating the variables influencing egg size. No significant effect of any of the variables on egg size was found (Table 23).

Table 23: Results of the GLM investigating the variables influencing egg size. *n*=27.

Explanatory variable	*df*	*t*	*P*
Cheek chromaticity of female's father	5,21	-1.110	0.279
Female standard length	5,21	1.440	0.165
Female condition	5,21	-0.130	0.898
Egg coloration	5,21	-1.243	0.227
Clutch size	5,21	-0.320	0.752

Excluding non-significant variables led to a null model undermining the results of the full model with none of the explanatory variables being significant.

B.3 Discussion

There was no effect at all of male coloration on egg chromaticity or the other egg characteristics of the daughter. While the intensity of the carotenoid-based breeding coloration is inherited by sons from their fathers (Bakker & Milinski, 1993), I could not find any genetic correlation between the father's ornament and the daughter's likelihood or ability to invest carotenoids into her eggs. The main factor influencing the egg coloration of a female's eggs thus seems to be the carotenoid content of her diet. Female preference for male breeding coloration has been shown to be genetically based (Bakker, 1993a). By preferentially choosing more intensely colored males for mating, females will have direct advantages because their mating partners will be better fathers for the offspring (Milinski & Bakker, 1990; Bakker & Milinski, 1993; Bakker & Mundwiler, 1994; Candolin, 1999; Pike *et al.*, 2007a; Pike *et al.*, 2009). Additionally the females gain indirect advantages by producing sons of higher quality, because fathers will pass on their coloration and good genes to the male offspring, while at the same time, the female offspring is not negatively affected by any antagonistically working genes correlated to carotenoid investment. However, female offspring does not seem to gain advantages connected to carotenoid investment by having a high quality father. In a norwegian stickleback population in which the females develop a carotenoid-based red coloration on their pelvic spines, a negative correlation was found between the red intensity of the spines and the carotenoid content of the females' gonads (Nordeide *et al.*, 2006). Males of the population have been shown to prefer females which do not show the conspicuous coloration or exhibit it less intensely (Nordeide, 2002). The authors raise the question why females with red pelvic spines are present in the population although not preferred by males. The redder females must have advantages that compensate for the cost of being less attractive to males. The authors mention several possible explanations including advantages of the red color in intraspecific interactions or higher inclusive fitness for red-spined females by having redder, potentially more attractive brothers. Considering the fact that females with redder spines have lower concentrations of carotenoids in their gonads, it might be possible that the offspring of these females benefit from the advantages found in terms of hatch rate and growth parameters in this study for offspring hatching from less

intensely colored eggs. If there is a genetic correlation between the mother's and the daughter's spine coloration – something which has not yet been investigated – the reduced egg carotenoid content and the subsequent fitness benefits might be another possible explanation for females with red spines being present in the population.

While egg chromaticity was not influenced by clutch size in 2010, this was found in 2009. As already mentioned earlier, mean egg chromaticity of the three different carotenoid diet group females of part A was compared to mean chromaticity of the eggs laid by the females investigated in part B. It was found that the latter ones were most similar to the zero and low carotenoid diet group. Thus, the eggs supposedly contained relatively little carotenoids and were colored accordingly. From this it can be concluded that if the effects of carotenoids on offspring fitness as found in part A are a general pattern in sticklebacks, the females in part B would have produced eggs and offspring with relatively high fitness in terms of hatching success and the investigated growth parameters. Effects of clutch size on egg coloration were only tested for the pooled data of all carotenoid diet groups in part A of my study. Since no significant effect was present, no further analyses were done within the diet groups. It might be possible that part B reveals a strategy present in the fish that was underestimated in the results of part A. Whether the finding of part B, that eggs are more intensely colored as clutch size rises, is a general pattern and if the effect could also be connected to experienced carotenoid level, has to be clarified by further within-group analyses with increased sample size for different carotenoid diet groups. Carotenoid investment (whether active or passive) into the eggs could depend on the general level of carotenoids females experience in their diet, e.g. rather on the lower or on the higher side of the natural range. It might be the case that different decisions are made or different physiological mechanisms work in dependence of experienced dietary carotenoid level and thus the connection between clutch size and egg coloration as found here would only be true for relatively low carotenoid concentrations of the diet. Of course the reported results could also be valid only for the investigated population or laboratory group of fish. Further investigation will be necessary to clarify these matters.

According to their coloration, carotenoid content of the eggs investigated in part B is placed on the lower side of the natural range, thus leading to a relatively high offspring fitness in terms of hatch rate and growth parameters (see part A). It might be the case that those eggs within the investigated group, which were more lightly colored than the average, or the eggs more intensely colored than the average, might not have been confronted with high further advantages or disadvantages, as offspring fitness was generally fairly high for the whole group. This might explain why the pattern of bigger females producing more eggs, and bigger clutches containing more intensely colored eggs, thus minimizing offspring fitness, could still be adaptive. This would be true as long as carotenoid content does not rise above a certain level where negative effects of carotenoids exceed the advantages gained by a higher clutch size. Stickleback males have been shown to prefer to mate with bigger females (Kraak & Bakker, 1998), and this is a pattern not only found in the stickleback but also in a lot of other fish species. If males would have chosen to mate with the bigger females of part B of my study, they would have gained bigger clutches, but also more yellowish colored clutches with all the consequent fitness disadvantages. However, the positive effects of gaining a high clutch size might be weighing more than negative effects of carotenoids in this carotenoid level range, where offspring fitness is still relatively high in comparison to eggs more intensely colored. Thus, by choosing the bigger females the males would still gain overall advantages, by obtaining clutches high in number of eggs, while the carotenoid-dependent fitness of the offspring would only be slightly lowered. If the same pattern of clutch size being dependent on egg color was found to be valid for the whole possible range of carotenoid amount in the diet/eggs, males might start to make different decisions in their mate choice or paternal investment as carotenoid level rises and negative effects of carotenoids on offspring fitness become more and more severe.

Although females originated from the same population (Texel) in part A and B of my study, I found some differences between the variables influencing egg characteristics.

In 2009, when investigating the explanatory variables for clutch size, egg size and egg coloration, the only effect found was that clutches with more eggs

were on average more intensely colored. In contrast, female traits such as standard length and condition proved to be influential on egg characteristics in 2010, while egg coloration was not related to clutch size.

Why are there discrepancies in the results from the 2009 and the 2010 experiment? Generally, there is the possibility of annual variation of reproductive characteristics occurring even within the same population (e.g. (Poizat *et al.*, 2002). Differences can also occur for example between breeding sides within one breeding season and population (e.g. (Bakker & Mundwiler, 1994) or between fish kept at different temperature regimes (e.g. (Ouellet *et al.*, 2001). Additionally, while the eggs for which egg coloration was measured were produced by wild-caught females in part A, in part B the egg laying females were the laboratory-raised F1 offspring of wild-caught fish. Females in part A were fed with artificial, carotenoid-enriched food, while the females in part B were fed a natural food source, namely chironomid larvae. The maintenance of the fish in different laboratories, the different rearing conditions and/or the different food regimes might explain why females showed different reproductive characteristics. It is especially interesting that in 2009, female traits like standard length and condition were not found to influence egg characteristics, since this is a usual pattern found in fish (e.g. winter flounder(Buckley *et al.*, 1991), stickleback: part A of this study, (Bakker & Mundwiler, 1994), common nase: (Keckeis *et al.*, 2000), Atlantic cod: (Ouellet *et al.*, 2001), plaice: (Kennedy *et al.*, 2007), marbled sole: (Higashitani *et al.*, 2007), reviewed in (Kamler, 2005). However, there was a trend of female standard length positively influencing clutch size, an effect which I also found in part A of my study. Most likely this effect would have become significant when analyses would have been repeated with a higher sample size in part B. Summarizing, while similarities can be found between the females of the 2009 and 2010 investigation in terms of maternal parameters affecting egg characteristics, there are also some differences which point out that even fish from the same population exhibit differences in reproductive characters when raised and kept under different conditions.

Conclusions

No sexually antagonistic genes or any other genetic correlation seem to be present in the three-spined stickleback determining the ability to invest carotenoids into (egg or breeding) ornaments. By choosing a high quality male exhibiting an intense red breeding coloration, a stickleback female seems to gain overall benefits for her offspring.

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6. Appendix

6.1 Variability of egg coloration

Figure 18. Samples of clutches of 30 stickleback females arranged according to average egg chromaticity (C^*_{ab}) of the clutch. The clutch with the least chromaticity is on the upper left, the one with the highest chromaticity on the lower right.

6.2 Survival rate in relation to original clutch size

For survival until the day one week after hatching, there was an only marginally significant effect of the interaction between female diet and water quality (Log likelihood test, Table 24) and therefore female diet and water quality were also investigated as main effects influencing hatch rate. Water quality was found to have a significant effect (Log likelihood test, Table 24). To be better able to compare the results of survival rates with the hatch rates, it was chosen to analyse the data for tap water and lake water separately despite of the interaction of water quality and female diet being only marginally significant. However, it must therefore be kept in mind to interpret the results with caution. Both of the

investigated covariates did not have a significant effect on survival (Log likelihood tests, Table 24).

Table 24: Results of the log likelihood tests investigating the variables influencing survival rate during

Explanatory variable	Δ d.f.	χ^2	*P*
Female carotenoid diet × water quality	1	3.009	0.083
Female carotenoid diet	1	1.795	0.180
Water quality	1	4.036	0.045
Clutch size	1	1.825	0.177
Average egg size	1	0.104	0.747

6.2.1 Survival rate in tap water

Hatchlings of the females of the three carotenoid diet groups did not exhibit different survival rates until the age of one week (Log likelihood test, Δ *d.f.*=1, χ^2=0.008, *P*=0.931, Figure 19).

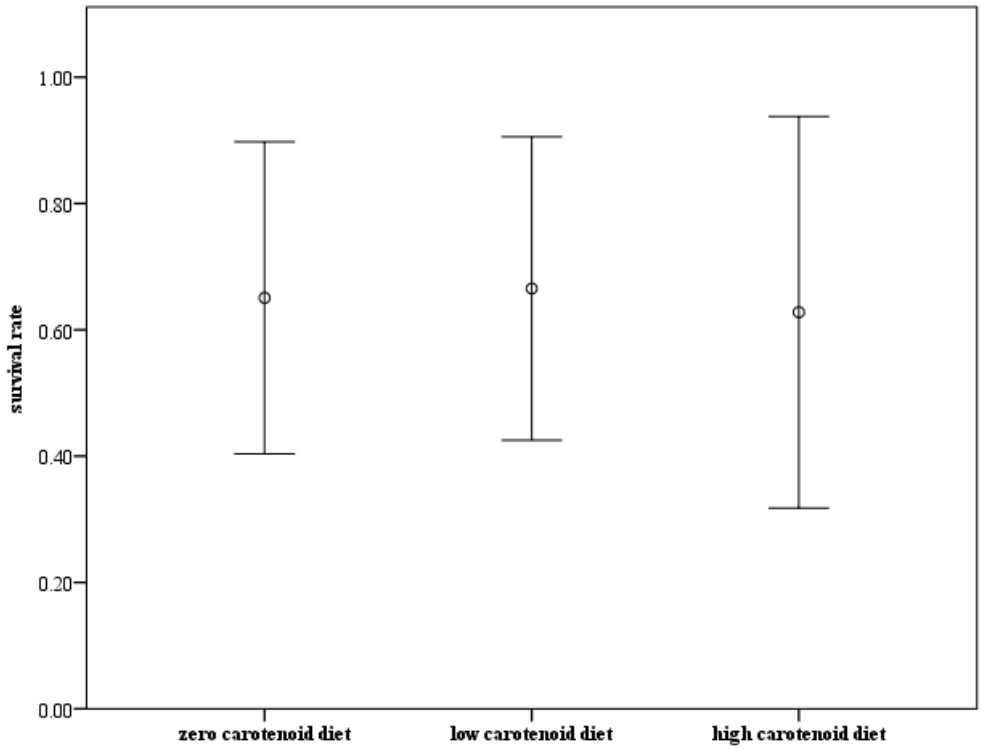

Figure 19. Different mean survival rate (± SD) of the clutches from the zero, low and high carotenoid diet females raised in tap water.

6.2.2 Survival rate in lake water

As for hatch rate, in lake water the survival rate of hatchlings deriving from the females receiving a zero carotenoid diet was significantly higher than the survival rate from the hatchlings of females fed a high carotenoid diet. Hatchlings deriving from the females of the zero and the low carotenoid diet females, and the low and high carotenoid diet females, did not differ significantly (Log likelihood tests, Table 25, Figure 20).

Table 25: Results of the log likelihood tests investigating the differences in mean survival rate between the newly hatched offspring of females of the different carotenoid diet treatments (n=42

	Δdf	χ^2	P
Zero carotenoid diet vs low carotenoid diet	1	3.324	0.068
Zero carotenoid diet vs high carotenoid diet	1	4.910	0.027*
Low carotenoid diet vs high carotenoid diet	1	0.162	0.688

Results reaching the significance level of P<0.05 are marked with a *

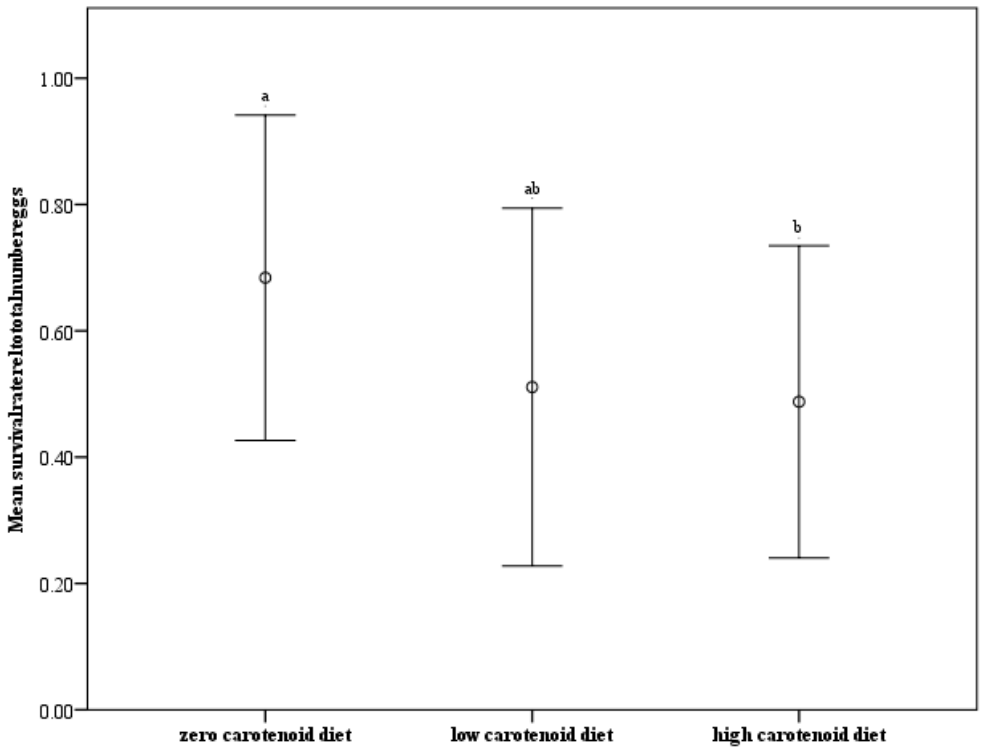

Figure 20. Different mean survival rate (± SD) of the clutches from the zero, low and high carotenoid diet females raised in lake water. Significant differences can be recognized by the bar annotations.

As mentioned above, these results have to be interpreted with caution, since the interaction effect of water quality and female diet was not significant and strictly speaking, the post hoc tests are therefore illegitimate.

Additionally, it must be noted that the patterns of survival rate of the hatchlings in tap or lake water are a product the differential hatch rates in the two raising environments because no significant differences were found in survival when comparing the six different test groups (tap water zero, low and high carotenoid eggs, lake water zero, low and high carotenoid eggs). (Kruskal Wallis, *d.f.*=5, χ^2=4.948, *P*=0.422). Survival rates in relation to original clutch size therefore only mirror hatch rates.

6.2.3 Female and male random effects on survival rate

Again, female identity was found to be significantly influencing the survival of the hatchlings until the age of one week after coming out of the egg (Log likelihood test, Δ *d.f.*=1, χ^2=5.691, *P*=0.017). Male identity did not have an

effect on the survival of the hatchlings (Log likelihood test, Δ *d.f.*=1, χ^2=1.550, *P*=0.100).

6.2.4 Female traits influencing the offspring's survival rate

Females which were in worse condition turned out to have offspring with higher survival rate (GLM, Table 26, Figure 19).

Table 26: Results of the general linar model investigating the female-dependent traits influencin survival rate (*n* = 42).

Explanatory variable	*df*	*t*	*P*
Female standard length	4,37	0.201	0.565
Female condition	1,40	-2.159	0.037*
Clutch size	4,37	-0.758	0.453
Average egg size	4,37	-0.008	0.994

Results reaching the significance level of *P*<0.05 are marked with a *

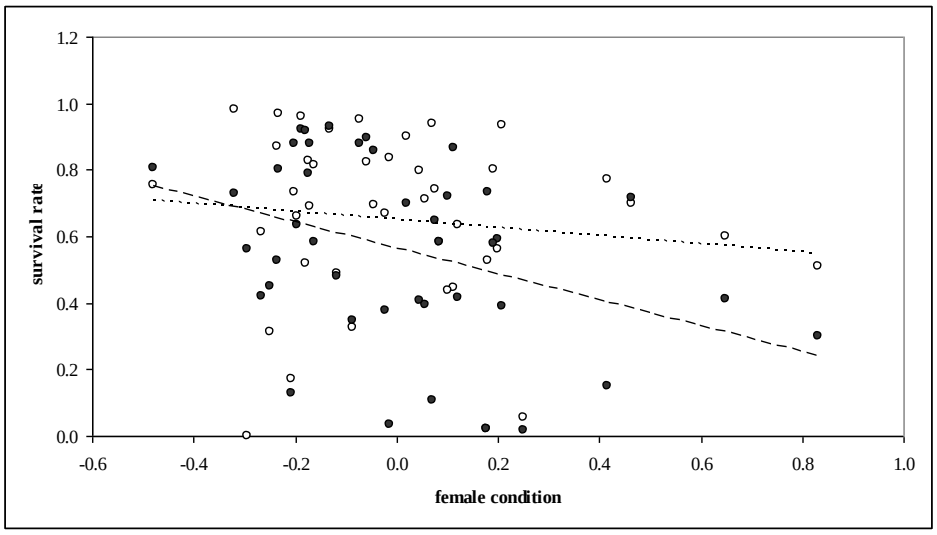

Figure 21. Correlation between female condition and offspring survival rate in the two different rearing environments (tap water open circles, upper dashed line, lake water black circles, lower dashed line). Survival rate is given as the number of fish in relation to original egg number surviving until one week after hatching. See text for statistics.

6.3 Statistical Results

The results of the statistical models (preliminary investigations) which are not mentioned in the *Results* of *Part A* and *B* are listed here.

6.3.1 Clutch size, egg size and fertilization rate

Table 27: Results of the general linear model investigating the traits influencing clutch size (n = 44).

Explanatory variable	*df*	*t*	*P*
Female treatment	5,38	-0.265	0.793
Female standard length	5,38	2.421	0.020*
Female condition	5,38	2.135	0.039*
Date breeding	5,38	-0.406	0.687
Average egg size	5,38	-2.865	0.007*

Results reaching the significance level of $P<0.05$ are marked with a *

Table 28: Results of the general linear model investigating the traits influencing egg size (n = 44).

Explanatory variable	*df*	*t*	*P*
Female treatment	5,38	-0.299	0.767
Female standard length	5,38	1.882	0.068
Female condition	5,38	1.704	0.097
Date breeding	5,38	-0.499	0.621
Clutch size	5,38	-2.865	0.007*

Results reaching the significance level of $P<0.05$ are marked with a *

Table 29: Results of the general linear model investigating the traits influencing fertilization rate (n = 44).

Explanatory variable	*df*	*t*	*P*
Female treatment	4,39	-0.822	0.416
Date breeding	4,39	0.247	0.806
Clutch size	4,39	-0.877	0.386
Average egg size	4,39	-0.208	0.836

6.3.2 Hatch rate

Table 30: Results of the linear mixed-effects model investigating the traits influencing hatch rate (n = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	20	0.619	0.543
Water quality	40	1.352	0.184
Female treatment*water quality	40	-2.385	0.021*
Date breeding	20	1.388	0.181
Clutch size	20	-2.137	0.045*
Average egg size	20	-1.606	0.124

Results reaching the significance level of P<0.05 are marked with a *

Table 31: Results of the linear mixed-effects model investigating the traits influencing hatch rate in tap water (n = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	20	0.606	0.551
Date breeding	20	1.430	0.168
Clutch size	20	-1.541	0.139
Average egg size	20	-0.905	0.376

Table 32: Results of the linear mixed-effects model investigating the traits influencing hatch rate in

Explanatory variable	*df*	*t*	*P*
Female treatment	20	-2.154	0.044*
Date breeding	20	0.784	0.443
Clutch size	20	-1.893	0.073
Average egg size	20	-1.685	0.108

Results reaching the significance level of P<0.05 are marked with a *

Table 33: Results of the general linear model investigating the female-dependent traits influencing hatch rate (n = 42).

Explanatory variable	*df*	*t*	*P*
Female standard length	4,37	0.298	0.768
Female condition	4,37	-2.972	0.005*
Clutch size	4,37	-1.167	0.258
Average egg size	4,37	-0.929	0.359

Results reaching the significance level of P<0.05 are marked with a *

6.3.3 Survival rate

Table 34: Results of the linear mixed-effects model investigating the traits influencing survival rate relative to clutch size (n = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	21	-0.137	0.892
Water quality	40	0.805	0.426
Female treatment*water quality	40	-1.702	0.097
Clutch size	21	-1.316	0.202
Average egg size	21	-0.312	0.759

Table 35: Results of the linear mixed-effects model investigating the traits influencing survival rate in tap water relative to clutch size (n = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	21	-0.028	0.935
Clutch size	21	-0.601	0.554
Average egg size	21	-0.661	0.516

Table 36: Results of the linear mixed-effects model investigating the traits influencing survival rate in lake water relative to clutch size (n = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	21	-2.128	0.045
Clutch size	21	-1.686	0.107
Average egg size	21	-0.239	0.813

Table 37: Results of the general linear model investigating the female-dependent traits influencing survival rate relative to clutch size (n = 42).

Explanatory variable	*df*	*t*	*P*
Female standard length	4,37	0.201	0.842
Female condition	4,37	-1.780	0.833
Clutch size	4,37	-0.758	0.453
Average egg size	4,37	-0.008	0.994

Table 38: Results of the linear mixed-effects model investigating the traits influencing survival rate relative to hatch rate (n = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	21	0.663	0.514
Water quality	39	0.066	0.948
Female treatment*water quality	39	-0.643	0.524
Clutch size	21	-0.940	0.358
Average egg size	21	1.338	0.195
Number of hatchlings	39	1.845	0.073

Table 39: Results of the general linear model investigating the female-dependent traits influencing survival rate relative to hatch rate (n = 42).

Explanatory variable	*df*	*t*	*P*
Female standard length	4,37	0.498	0.621
Female condition	4,37	-0.234	0.816
Clutch size	4,37	1.057	0.298
Average egg size	4,37	-0.008	0.994

6.3.4 Offspring size and growth parameters

Table 40: Results of the linear mixed-effects model investigating the traits influencing offspring size 6 weeks after hatching (n = 42).

Explanatory variable	*df*	*t*	*P*
Water quality	36	-2.676	0.011*
Female treatment	18	-1.794	0.090
Water quality*female treatment	36	1.959	0.058
Raising density	36	-2.521	0.016*
Date breeding	18	3.704	0.002*
Average egg size	18	2.029	0.058

Results reaching the significance level of P<0.05 are marked with a *

Table 41: Results of the linear mixed-effects model investigating the traits influencing offspring size 6 weeks after hatching in tap water (n = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	17	-1.979	0.064
Raising density	17	0.500	0.624
Date breeding	17	3.327	0.004*
Average Egg size	17	3.052	0.007*

Results reaching the significance level of P<0.05 are marked with a *

Table 42: Results of the linear mixed-effects model investigating the traits influencing offspring size 6 weeks after hatching in lake water (n = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	17	-0.034	0.973
Raising density	17	-2.802	0.012*
Date breeding	17	3.282	0.004*
Average Egg size	17	0.981	0.340

Results reaching the significance level of P<0.05 are marked with a *

Table 43: Results of the linear mixed-effects model investigating the traits influencing initial growth rate *b* of the hatchlings (*n* = 42).

Explanatory variable	*df*	*t*	*P*
Water quality	38	1.218	0.231
Female treatment	38	-0.521	0.605
Water quality*female treatment	38	-2.110	0.042*
Raising density	38	-1.889	0.067
Date breeding	38	0.122	0.904

Results reaching the significance level of $P<0.05$ are marked with a *

Table 44: Results of the linear mixed-effects model investigating the traits influencing initial growth rate *b* of the hatchlings in tap water (*n* = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	37	-0.475	0.637
Raising density	37	-1.628	0.112
Date breeding	37	0.650	0.520

Table 45: Results of the linear mixed-effects model investigating the traits influencing initial growth rate *b* of the hatchlings in lake water (*n* = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	37	-2.628	0.012*
Raising density	37	-0.919	0.364
Date breeding	37	-0.575	0.569

Results reaching the significance level of $P<0.05$ are marked with a *

Table 46: Results of the linear mixed-effects model investigating the traits influencing growth rate change *c* of the hatchlings (*n* = 42).

Explanatory variable	*df*	*t*	*P*
Water quality	38	-1.781	0.083
Female treatment	21	0.315	0.756
Water quality*female treatment	38	2.528	0.016*
Raising density	38	1.080	0.287
Date breeding	21	0.955	0.350

Results reaching the significance level of $P<0.05$ are marked with a *

Table 47: Results of the linear mixed-effects model investigating the traits influencing growth rate change *c* of the hatchlings in tap water (*n* = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	20	0.312	0.759
Raising density	20	1.840	0.081
Date breeding	20	0.208	0.838

Table 48: Results of the linear mixed-effects model investigating the traits influencing growth rate change *c* of the hatchlings in lake water (*n* = 42).

Explanatory variable	*df*	*t*	*P*
Female treatment	20	2.840	0.001*
Raising density	20	-0.294	0.772
Date breeding	20	1.490	0.152

Results reaching the significance level of $P<0.05$ are marked with a *

7. Zusammenfassung

Elterntiere können die Qualität ihrer Nachkommen einerseits auf indirektem Weg über die Gene, andererseits auf direktem Weg, wie zum Beispiel über maternale Effekte beeinflussen. Maternale Effekte, bei denen aufgrund von genetischen oder umweltbedingten Unterschieden in der maternalen Generation phänotypische Unterschiede in der Nachkommengeneration entstehen, spielen besonders in frühen Lebensstadien häufig eine wichtige Rolle und wurden im Speziellen in eierlegenden Spezies untersucht, wo die Mütter ihren Nachkommen im Ei verpackt alle lebenswichtigen Stoffe mitgeben. Carotenoide sind einer jener Inhalte, die häufig in Eiern sowohl bei Vögeln als auch bei Fischen gefunden werden. Es wird weitläufig angenommen dass Carotenoide eine Reihe von positiven Eigenschaften besitzen, zum Beispiel die Wirkung als Antioxidantien oder in der Immuno-Regulation und Immuno–Stimulation. Carotenoide spielen im Tierreich aber noch eine andere tragende Rolle, indem sie bei vielen Arten für die Ausprägung von gelber bis roter (Signal)färbung verantwortlich sind, so auch für die typische rote Färbung des männlichen dreistacheligen Stichlings *Gasterosteus aculeatus* während der Brutsaison. Die Intensität der Färbung spiegelt die Qualität der Stichlingsmännchen wieder und ist somit ein so genanntes ehrliches Signal. Männchen vererben die Intensität ihrer Rotfärbung an ihre Söhne. Bei vielen Fischarten ist aufgrund chemischer Analysen bekannt, dass ihre Eier Carotenoide enthalten. Ein möglicher Zusammenhang zwischen Intensität von beobachteter Eifärbung und Carotenoidgehalt in Fischeiern wurde allerdings bisher wenig untersucht. Jedoch gerade bei Fischen mit ihrer transparenten Eihülle, die Färbung und somit möglicherweise Carotenoidgehalt sichtbar macht, könnte die Färbung der Eier eine tragende Rolle für parentales Investment spielen. Dies wäre dann der Fall, wenn unterschiedlicher Carotenoidgehalt auf unterschiedliche Ei- und somit Nachkommenqualität hinweist.

In meiner Studie wurde untersucht, ob sich bei Stichlingen die Zufütterung von unterschiedlichen Carotenoid-Mengen in der maternalen Generation auf das Investment von Carotenoiden in die Eier – gemessen anhand der Eifärbung – auswirkt. Zweitens wurde getestet, ob (wenn vorhanden) das unterschiedliche Carotenoid-Investment in die Eier Auswirkungen auf die Fitness der

Nachkommen hat. Drittens wurde untersucht ob Stichlingsmännchen, die wie erwähnt ihre Fähigkeit Carotenoide in Ornamente zu investieren an ihre Söhne weitergeben, dies auch an ihre Töchter vererben (was sich in dem Investment von Carotenoiden in die Eier niederschlagen könnte), oder ob je nach Geschlecht antagonistisch wirkende Gene vorhanden sind, sodass Söhne eines attraktiven Vaters zwar profitieren, Töchter aber einen Nachteil haben.

Um die ersten beiden Fragen zu klären wurden drei unterschiedliche Diätgruppen etabliert, in denen Stichlingsweibchen mit Futter das entweder gar keine, eine niedrige (40mg/kg Futter) oder eine hohe (200mg/kg Futter) Menge an Carotenoiden enthielt gefüttert wurden. In Folge wurde die Eifärbung in den unterschiedlichen Diätgruppen gemessen, und die Nachkommen der jeweiligen Weibchen zur einen Hälfte in einer sterilen und zur anderen Hälfte in einer naturnahen Umwelt (Leitungswasser versus Teichwasser) aufgezogen. Die Schlupfrate, Überlebensrate und Wachstumsrate der Nachkommen wurden als Fitnessindikatoren dokumentiert. Um die dritte Frage bezüglich der möglicherweise antagonistisch wirkenden Gene zu beantworten, wurden Fischfamilien über drei Generationen hinweg untersucht und die Intensität der Brutfärbung der Männchen in Zusammenhang mit der Eifärbung ihrer Töchter gestellt.

Die Menge an Carotenoiden in der Diät der Mütter wirkte sich auf die Eifärbung aus, wobei die Diät ohne und mit wenigen Carotenoiden ähnliche Ergebnisse brachte, während die Intensität der Gelbfärbung der Eier von Weibchen die viele Carotenoide in ihrer Diät erhielten deutlich höher war. Bezüglich der getesteten Fitnessparamter der Nachkommen ergab sich, dass die Umwelt in der die Jungfische aufwuchsen einen maßgeblichen Einfluss auf die Effekte von Carotenoiden hatte. Die Schlupfrate der Fische in Leitungswassser war unabhängig vom Carotenoidgehalt in der Diät der Mutter, wohingegen in Teichwasser zunehmender Carotenoidgehalt der maternalen Diät zu einer reduzierten Schlupfrate führte. Im Teichwasser groß gezogene Fische wiesen außerdem unterschiedliche Wachstumskurven auf: Nachkommen der Mütter mit hoher Carotenoiddiät wuchsen in den ersten Lebenswochen langsamer als die Nachkommen der Mütter die weniger Carotenoide erhalten hatten, konnten diesen Rückstand allerdings später durch schnelleres Wachstum wieder aufholen. Im Leitungswasser hingegen ergab sich kein Effekt von Carotenoidgehalt in der

Diät der Mutter auf die Wachstumskurven der Nachkommen. Die Intensität der Paarungsfärbung eines Männchens hatte weder einen positiven noch einen negativen Einfluss auf das Carotenoidinvestment der Tochter in ihre Eier.

Meine Arbeit zeigt, dass die Gelbfärbung der Eier tatsächlich vom Carotenoidgehalt der Diät der Mutter abhängt und mit zunehmender Intensität einen negativen Einfluss auf die gemessenen Fitnessparameter von Nachkommen hat, die unter naturnahen Bedingungen aufwachsen.
Das Auftreten von antagonistisch wirkenden Genen wurde nicht gefunden. Hervorzuheben ist besonders das Ergebnis, dass Carotenoide auch innerhalb der Dosierungen in der sie in der Natur vorkommen negative Effekte haben können, was im Gegensatz zu bisher durchgeführten Arbeiten über die Auswirkungen von Carotenoiden steht, die zum Großteil über positive oder ein Fehlen von Effekten berichten. Zudem wurde festgestellt, dass sterile Laborbedingungen offensichtlich nicht immer ausreichend sind um diverse mögliche Auswirkungen von Carotenoiden in lebenden Organismen detektieren zu können. Vor diesem Hintergrund muss vor allem jene früheren Studien, die unter Laborbedingungen durchgeführt wurden und keine oder positive Effekte von Carotenoiden festgestellt haben, neu bewertet werden.

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9. Curriculum vitae

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12/2009 - 11/2011	Diploma thesis at the Konrad Lorenz Institute for Ethology; Theme: The evolutionary significance of carotenoid-mediated egg coloration in the three-spined stickleback *Gasterosteus aculeatus*.
1/2009 - 6/2009	Erasmus-scholarship at the Department of Animal Science of the University of Aarhus, Denmark
10/2004 - 12/2011	Studies of Biology/Zoology at the University of Vienna, Austria
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10/2003 - 6/2006	Scandinavian Studies at the University of Vienna, Austria
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Work experience

10/2009 - today	Physician assistant in the team of Dr. med. Rudolf Ganger, orthopaedist, Vienna.
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