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CAROTENOID-BASED MALE SEXUAL ORNAMENTATION IN THE REDSIDE DACE (*Clinostomus elongatus*)

By

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DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is the result of joint research. My data chapter was co-authored with my supervisor, Dr. Trevor Pitcher, and with Dr. Stéphanie Doucet. My advisor and collaborator provided valuable feedback, helped with the project design and statistical analyses, and provided editorial input during the writing of my manuscript. However, the primary contributions have been by the author. Chapter 2 has been prepared as a manuscript, and will be submitted to Functional Ecology for publication.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from my co-authors to include the above materials in my thesis. I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work, completed during my registration as a graduate student at the University of Windsor.

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ABSTRACT

Male sexual ornamentation may represent a signal designed to increase male reproductive success by conveying information to females. Carotenoid-based ornaments may serve as particularly interesting signals because of the unique properties of carotenoids. I investigated allocation issues, the life-history trade-off and reliable signalling hypotheses, and the source of carotenoid-based colouration in a wild population of male redside dace (*Clinostomus elongatus*). I identified the chemical basis of the red integument as keto-carotenoids, used reflectance spectroscopy to obtain the spectral properties of the red colouration to test the reliable signalling hypothesis, used absorbance spectroscopy readings to quantify carotenoid content in different tissues to test for allocation and trade-offs, and identified the natural source of the carotenoids. My findings indicate that the red integument is not a reliable signal, no trade-off was observed across tissue types, and terrestrial insects were the natural source of carotenoids for redside dace.

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CHAPTER 1: GENERAL INTRODUCTION

Sexual Selection

The existence of conspicuous sexual ornamentation appears to be in conflict with Darwin's theory of natural selection because ornamentation ought to impede survival (Andersson 1994). In order to rectify this, Darwin proposed the concept of sexual selection which is a male's struggle not for survival, but a struggle to increase reproductive success (Darwin 1871, Andersson 1994). Darwin proposed that male ornaments evolved through sexual selection by female choice and male armaments or weapons, evolved through sexual selection with direct competition between males for access to females (Andersson 1994).

There are three different categories to describe characteristics that differ between males and females, as outlined by Darwin. They are primary sex traits, secondary sex traits, and ecological traits (Andersson 1994). Primary sex traits are those that are directly related to reproduction such as gonad size. Secondary sex traits are not directly related to reproduction but yet still vary between males such as body size or an elaborate male ornament. Ecological traits are related to habits that may differ between the sexes, for example the different feeding habits of male and female mosquitoes in which females feed on blood and males feed from flowers (Andersson 1994). Sexual selection serves as a theory that can explain the often elaborate and conspicuous secondary sexual traits observed across taxa (Andersson 1994).

There are several hypotheses that attempt to explain the reasons that females are choosy specifically with respect to ornamentation. First, Fisher's theory of runaway selection states that females prefer males with a trait that offers a slight natural selection

advantage; this female preference spreads throughout the population and drives the trait to become more elaborate in males (Fisher 1930). Second, sexual conflict through the chase-away selection hypothesis states that a male will develop a mutation that taps into a pre-existing preference in some females in the population, as some females become resistant to this exploitative trait; males evolve even more elaborate forms of the trait in order to gain their preference (Holland & Rice 1998). Third, the good genes hypothesis states that it would be advantageous for females to mate with males with elaborate ornamentation because this is an indication of their superior genes that can improve the genetic quality of their offspring (Zahavi & Zahavi 1975). Finally, the healthy male hypothesis states that only males in good health and without parasites can afford to display elaborate ornamentation and therefore, females will not be exposed to illness or parasites by mating with these males (Hamilton & Zuk 1982). A common form of ornamentation that has been linked to female choice across taxa is a display of colouration (Waitt et al. 2003, Houde & Endler 1990, Hill 1991). This colouration can often be explained mechanistically as deposits of carotenoid molecules in the feathers, integument, or other tissues of organisms (Olson & Owens 1998).

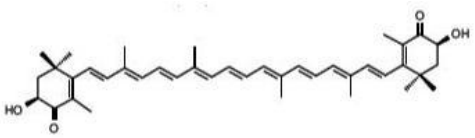
Carotenoid properties and ornamentation

Carotenoids were first recognized in 1837 as alcohol solvent molecules that produced the yellow colouration in dried leaves and they were named xanthophylls (Goodwin 1954). Further analysis of these molecules revealed that they consisted of two separate groups: molecules soluble in hydrocarbons were named carotenes and molecules soluble in ethanol were named xanthophylls (Goodwin 1954). Carotenoids were defined

by Karrer in 1931 as, “yellow to red pigments of aliphatic or alicyclic structure, composed of isoprene units (usually eight) linked so that the two methyl groups nearest the centre of the molecule are in positions 1:6 whilst all other lateral methyl groups are in positions 1:5; the series of conjugated double bonds constitutes the chromophoric system of the carotenoids” (Goodwin 1954). The molecular structure of carotenoids is responsible for their unique functions for various reasons (Britton et al. 2008).

Carotenoids are three-dimensional molecules, can exist in different geometric forms (cis/trans), and most are chiral (Britton et al. 2008). The key element of most carotenoids is the carbon double bond chain that occurs centrally within the molecule (Britton et al. 2008, See Fig 1.).

Figure 1: Carotenoid astaxanthin molecular structure (adapted from Britton et al. 2008)



The π-electrons in this double bond system are highly delocalized and the energy required to transition the molecule to its low-energy excited state is very low. This energy also corresponds to visible light in the region of 400-500nm which is the reason that carotenoids exhibit their characteristic yellow to red colour (Britton et al. 2008). When mixed with proteins, however, carotenoids may also appear green, blue or purple (Olson & Owens 1998). The centrally located, electron-rich conjugated double bond system within the molecule is susceptible to attack by electrophiles and oxidizing free radicals, which is why carotenoids are generally labeled as antioxidants (Britton et al. 2008).

Many studies have been conducted that confirm the existence of carotenoid-based ornamentation. In three species of widowbirds (*Euplectes ardens), (Euplectes macrourus),* and *(Euplectes axillaris);* red and yellow plumage colouration has been attributed to specific carotenoids using reflectance spectrometry and high performance liquid chromatography. Yellow colouration was found to be derived from lutein, zeaxanthin, as well as other xanthophylls. Red colouration was found to be derived from 4-keto-carotenoids most commonly α- and β-doradexanthin and canthaxanthin or lutein, zeaxanthin and anhydrolutein (Andersson et al. 2007).

Experimental manipulations of diet have also been used to confirm the link between carotenoids and colouration. In goldfinches (*Carduelis tristis),* the diet of yellow birds was supplemented with red carotenoids in order to determine the effect this would have on plumage colouration. In northern cardinals (*Cardinalis cardinalis),* red carotenoids were removed from the diet to determine the effect on plumage colouration. It was found that the normally yellow goldfinches displayed a new orange colouration and the normally red cardinals displayed pale red colouration therefore confirming the importance of carotenoids to the colouration of these species (McGraw et al. 2001).

Reliable Signalling hypothesis

In nature, a signal is defined as an act or structure generated by a sender to increase their fitness by invoking a behaviour in the receiver, in turn the receiver attempts to interpret the signal to increase their own fitness (Espmark et al. 1998). A signal is considered reliable if: (i) a characteristic of the signal is correlated with an attribute of the signaller or the signaller's environment and (ii) the receiver will benefit from having

information about this attribute (Searcy & Nowicki 2005). A carotenoid-based ornament may be a reliable signal if the spectral properties of the colouration of the male's ornament are correlated with the carotenoid concentration of the ornament and if females benefit from knowing the carotenoid concentration of the male's ornament (Searcy & Nowicki 2005). Carotenoids can represent a male attribute about which females may benefit from having information because they may indicate that the male has adequate nutrition and elevated foraging skills, they may indicate that the male is not infected with parasites, and they may be costly to allocate to ornamentation and therefore honestly advertise the male's genetic quality (Searcy & Nowicki 2005, Olson & Owens 1998, Smith & Harper 2003).

There is empirical evidence that links acquisition of elevated carotenoid levels and maintenance of highly developed carotenoid-based ornaments with superior genetic quality in males. For example, in a study conducted on three-spined sticklebacks (*Gasterosteus aculeatus)*, males were fed carotenoid supplemented diets at levels that were biologically relevant and control males were not supplemented. Split-clutch *in vitro* fertilization trials showed that males with the carotenoid supplemented diet had higher levels of functional fertility as well as a more highly expressed ornament than the control males (Pike et al. 2009). In a study of captive male greenfinches, males with brighter yellow feathers in which the colouration was carotenoid based, were found to have stronger immune reactions against newly introduced antigens (Saks et al. 2003). This indicates that carotenoid-based ornamentation is an honest signal of immunocompetence and health in this species.

The aforementioned hypotheses and examples support the idea that the display of a carotenoid-based ornament can be a signal to females of male quality. However, there is also evidence of deception in signalling systems (Searcy & Nowicki 2005). For example, in male green frogs (*Rana clamitans*), larger males are able to better defend territory and larger males also emit calls with lower frequency than smaller males. Smaller males have been observed lowering the frequency of their calls in order to give the impression that they are larger to help with territory defense (Bee et al. 2000). This raises the question of whether or not it is possible for deception to exist in carotenoid-based ornaments through lower quality males enhancing their ornament by making a trade-off between somatic carotenoids and carotenoids in their ornament in order to deceive females.

Life-History Trade-off hypothesis

The life history trade-off hypothesis states that when resources are scarce, an allocation trade-off may be required between reproduction and survival (reviewed in Zera & Harshman 2001). Adapting this hypothesis to apply to carotenoids would state that increasing allocation of carotenoids to either ornamentation or somatic physiological functions (immunity, tissue maintenance) decreases the carotenoid availability to the other function (Lin et al. 2010). For example, a study conducted on the gold morph and barred morph of Midas cichlids (*Amphilophus citrinellus*) tested the carotenoid trade-off hypothesis in terms of whether supplemented carotenoids would be allocated to ornamentation or to address an immune challenge. It was found that the gold morph allocated more carotenoids to their ornament while the barred morph sequestered more

carotenoids in the circulating plasma. However, the two morphs did not differ in innate immunity following the immune challenge (Lin et al. 2010). If a low quality male wanted to deceive females into believing he was of high quality, he could in theory draw from somatic carotenoids and allocate them to the ornament instead. A study conducted on male three-spined sticklebacks in the field found that ornamentation was significantly correlated with body condition (Barber et al. 2000). This study suggests that males of high quality were able to better support their ornamentation and does not provide support for a life history trade-off with respect to carotenoids occurring in wild sticklebacks.

Redside dace

The redside dace (*Clinostomus elongatus)*, is a small cyprinid with a red integument ornament that is presumably carotenoid-based and is therefore an ideal mating system for testing the reliable signalling and the trade-off hypotheses. The redside dace is found in small headwater streams in pool and riffle habitats and is most commonly captured in shaded areas under overhanging vegetation or near submerged branches and logs (McKee & Parker 1982). The distribution of the redside dace is discontinuous and they appear in the United States in headwater streams of the Great Lakes and the Mississippi River between Minnesota and New York (Novinger & Coon 2000). In Canada, redside dace occur only in Southern Ontario in tributaries of Lake Ontario, Lake Erie, and Lake Huron (Parker et al. 1988).

Redside dace are found only in very clear water and are thought to have strict water quality requirements (McKee & Parker 1982). It appears that redside dace are transient and do not spawn in the same streams they inhabit during the majority of the

year. For the spawning season, typically in late May, redside dace travel to parts of the stream with riffles and sand or gravel bottoms and spawning occurs at temperatures between 16°C and 19°C (McKee & Parker 1982). The redside dace is declining in most of its range and this is attributed in part to erosion and human activities such as construction which interferes with their water quality requirements (Novinger & Coon 2000).

Redside dace generally range from 23mm to 78mm in standard length and from 0.1g to 8.5g in mass for ages 0 – 3+ years (McKee & Parker 1982). Spawning occurs at 2+ years in the redside dace (Parker et a. 1988). The larger upturned mouth of the redside dace is suitable for catching terrestrial prey flying above the water's surface and studies have confirmed that the majority of the redside dace diet is terrestrial insects (Schwartz & Norvel 1958, Daniels & Wisniewski 1994).

Male redside dace have a black dorsal end with a yellow lateral band directly below. A red lateral band occurs directly below the yellow band, becomes black at the midline and ends at the posterior end. Another black lateral band begins at the anterior end and continues to the end of the head. The ventral end is silver in colour (Parker et al. 1988). Redside dace have a non-resource based mating system and sexual dimorphism in terms of the red integument which is visually more red in males than females. A study using reflectance spectroscopy confirmed that male and female redside dace have significantly different spectral properties (i.e. sexual dichromatism) for the red integument (Beausoleil 2009). As such, redside dace are an excellent species to study reliable signalling and the trade-off hypothesis with respect to their presumably carotenoid-based red integument ornamentation.

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CHAPTER 2: ALLOCATION PATTERNS, LIFE-HISTORY TRADE-OFF, AND RELIABLE SIGNALLING IN CAROTENOID-BASED ORNAMENTATION IN MALE REDSIDE DACE

Synopsis

Elaborate male ornamentation may signal reliable information to females for the purpose of mate choice. In order for a signal to be reliable, however, a characteristic of the signaller must be correlated with an attribute of the signaller or their environment and information about this attribute must be beneficial to the receiver. Because of their scarcity, conspicuousness, and somatic physiological functions, carotenoid-based ornaments may constitute reliable signals if carotenoid concentration is correlated to some characteristic of the signaller. I investigated the chemical basis of the red integument, carotenoid allocation, the reliable signalling hypothesis, the life-history trade-off hypothesis, and the natural source of carotenoids for the redside dace (*Clinostomus elongatu*s). I identified keto-carotenoids as the source of the colouration in the red integument. Male redside dace allocated significantly different amounts of carotenoids in terms of both carotenoid concentration and total carotenoid content to red, black, silver, and yellow integument, as well as muscle. No trade-off was detected, as males with more carotenoids in the red integument also had more carotenoids in the other integument types. A significant positive relationship was observed when comparing carotenoid content in the muscle to carotenoid content of all integument types combined and to carotenoid content of the black, silver, and yellow integument combined. A positive trend was also observed when comparing carotenoid content in the muscle with carotenoid content in the red integument, providing further evidence that no life-history trade-off occurred. I did not find a relationship between the spectral properties obtained

from reflectance spectroscopy and carotenoid concentration of the red integument and therefore could not confirm the reliability of the colouration as a signal. This study is the first to confirm the chemical basis of the red integument of the redside dace and offers insight into carotenoid allocations and life history trade-offs in a wild population.

Introduction

Two theories that explain the diversity in phenotypes seen in natural populations are natural selection, which is driven by an individual's attempt to survive and sexual selection, which is driven by an individual's attempt to reproduce (Andersson 1994). Natural selection typically selects for phenotypes that provide a direct link to survival such as camouflage or crypsis (Endler 1980). In contrast, the phenotypes selected for by sexual selection provide a direct link to reproduction and tend to be more conspicuous (Andersson 1994). Sexual selection comprises armaments that males typically possess to physically compete for females and ornaments that males typically possess in order to attract choosy females (Andersson 1994). The existence of armaments makes sense in light of evolutionary theory because they are clearly linked to the individual's reproductive success through his ability to challenge other males for access to females (Darwin 1871, Andersson 1994). However, the evolution of male ornamentation is more controversial because of their conspicuousness and lack of practical function. Because of this, ornaments would appear to reduce an individual's ability to survive and therefore, oppose natural selection (Darwin 1871, Andersson 1994).

There are four main hypotheses to explain the origin or existence of ornamentation. First, the run-away selection hypothesis proposes that the initial

evolution of sexual ornamentation can occur when traits that offer a slight natural selection advantage are selected for through female choice and therefore become prominent throughout the population (Fisher 1930). Once a trait becomes a target of female choice, the degree of prominence of that trait as well as the female preference for that trait evolve in unison until the point that the trait disrupts the balance between sexual selection and natural selection (Fisher 1930, reviewed in Andersson 1994). In the widowbird (*Euplectes progne)*, the male ornament is the tail as females prefer males with longer tails. A study was conducted with three treatment groups: males with their tails shortened, control males, and males with their tails lengthened using the feathers removed from the first treatment group. It was found that females preferred the males with the lengthened tails more than the control males and preferred the control males more than the males with the shortened tails (Andersson 1982). This is consistent with run-away selection because as the males developed larger ornaments, females also developed a preference for the more elaborate ornamentation. Second, the chase-away selection hypothesis proposes that males develop a novel trait that taps into a pre-existing sensory bias in females. This new female preference results in females accepting suboptimal mating conditions and therefore, females develop resistance to the trait to remedy this. This causes males to try to attract the resistant females by producing more elaborate versions of the same trait (Holland & Rice 1998). A study was conducted on two species of wolf spiders that differ because male *Schizocosa ocreata* have a large tuft of bristles on their forelegs and male *Schizocosa rovneri* do not. Female wolf spiders of both species were shown videos of males in which tufts were added, removed, or enhanced and were monitored for behaviours indicating reproductive receptiveness. In

female *S. ocreata*, there was no difference in receptiveness between the males with tufts, without tufts, or with enlarged tufts. However, in female *S. rovneri*, the addition of tufts doubled female receptiveness. This is indicative of both female resistance to a trait in *S. ocreata* and female sensory bias for a novel trait in *S. rovneri* (McClintock & Uetz 1996). Third, the good genes hypothesis states that males indicate their genetic quality through the degree of ornamentation they are able to produce and therefore females choose males in order to improve the genetic quality of their offspring (Zahavi & Zahavi 1975, reviewed in Andersson 1994). In a study conducted on peacocks (*Pavo cristatus)*, male-female pairs were created with males with elaborate trains and males with less elaborate trains. It was found that the offspring of the more ornamented males grew and survived better in a semi-natural enclosure which supports the good genes hypothesis (Petrie 1994). Finally, the healthy male hypothesis states that females choose males with elaborate ornaments because unhealthy males or males with parasites would not be able to maintain the ornament (Hamilton & Zuk 1982). Therefore, the female will not be exposed to illness by mating with a male with an elaborate ornament. In a study conducted on bowerbirds (*Ptilonorhynchus violaceus)*, it was found that values of the spectral property brightness of the plumage colour of adult and juvenile males were negatively correlated with blood parasites. Therefore, males that were infected with parasites were not able to produce equally bright plumage as males that were not infected with parasites (Doucet & Montgomerie 2003). Ornamentation is present in nature in many different forms and one of the most common examples observed across taxa is yellow or red colouration that is often the result of carotenoid deposits (reviewed by Olson & Owens 1998).

Carotenoid-based ornaments are widely studied because of their unique properties and the implications of allocating them to ornamentation. Carotenoids are hydrophobic molecules that exhibit centrally located conjugated chains of double bonds and polyisoprenoid structures (Britton 1995). As a result of the energy requirement to transition the molecule through electron exchange along the chain of conjugated double bonds, carotenoids appear yellow, orange, and red because the energy corresponds to light in the visible spectrum between 400 nm and 500 nm (Britton 1995). Recently, emphasis has been placed on assessing the molecules that produce the colour displayed in ornamentation in order to better understand the evolution of male secondary sexual characters and a carotenoid basis has been observed across taxa including but not limited to birds, reptiles, and fish (e.g. Hill 1991, Macedonia et al. 2000, Wedekind et al. 1998). For example, in house finches (*Carpodacus mexicanus)*, plumage colouration varies from pale yellow to vivid red and it has been shown that plumage colouration corresponds to dietary carotenoid intake (Hill 1991). In the Jamaican radiation of *Anolis* lizards, carotenoids were among the molecules isolated from the ornament, a coloured dewlap (Macedonia et al. 2000). In three-spined stickleback (*Gasterosteus aculeatus)*, the ornament is colouration on the throat which was determined to be related to the carotenoid content (Wedekind et al. 1998).

Because carotenoid-based ornaments are thought to affect female choice, they may be considered signals from male senders to female receivers (Searcy & Nowicki 2005). In order for a signal to be considered reliable, it must meet two criteria. First, a characteristic of the signaller must be consistently correlated with an attribute of the signaller or their environment (Searcy & Nowicki 2005). Second, the receiver must

benefit from receiving information about the attribute (Searcy & Nowicki 2005). Carotenoids are representative of attributes that females benefit from knowing about for various reasons. First, they may indicate elevated foraging skills or nutrition (Searcy & Nowicki 2005). In a study conducted on male guppies (*Poecilia reticulata*), there were two treatment groups, males fed a diet supplemented with carotenoids and males fed a diet absent of carotenoids. It was found that the males with the carotenoid supplemented diet has more strongly expressed orange colouration and were preferred by females (Kodric-Brown 1989, Searcy & Nowicki 2005). Second, carotenoid-based ornaments may indicate a lack of parasites (Searcy & Nowicki 2005). In great tits (*Parus major*), yearlings with greater spectral values of hue for their colouration and more lutein had increased survivorship and decreased blood parasitism (Horak et al. 2001). Third, carotenoid-based ornaments are costly to produce and may signal male quality (Searcy & Nowicki 2005).

Carotenoid-based ornaments are costly to produce and maintain for several reasons. First, because carotenoids cannot be produced de novo, additional foraging time may be required to obtain enough to produce an ornament (Searcy & Nowicki 2005, Olson & Owens 1998). Second, carotenoids have important somatic physiological functions and allocating them to ornamentation diverts them from being used in somatic tissue (Searcy & Nowicki 2005, Olson & Owens 1998). Third, carotenoids are difficult to digest and metabolize into an ornament (Searcy & Nowicki 2005, Olson & Owens 1998). Finally, the conspicuous colouration of carotenoid-based ornaments may make an organism more vulnerable to predation (Searcy & Nowicki 2005, Smith & Harper 2003). Because of these properties, carotenoid concentration represents an attribute about males

that females will benefit from knowing. Additionally, in order for a carotenoid-based ornament to be reliable, a characteristic of the signaller must be correlated with carotenoid concentration (Searcy & Nowicki 2005). Therefore, in order to test the reliability of carotenoid-based ornaments as signals, the spectral properties of the colouration of the ornament must correlate with the carotenoid concentration of the ornament.

In order to test the reliable signalling hypothesis in terms of the spectral properties, however, it must be known that the receiver is able to distinguish between different spectral properties. In teleost fishes for example, the eye morphology includes cones that respond to visible light from the ultraviolet to the red range, and therefore an appropriate method of testing the reliable signalling hypothesis in terms of spectral properties in teleost fishes, is monitoring reflected light between the wavelengths of 300 nm and 700 nm (Kusmic & Gualtieri 2000).

The life history trade-off hypothesis states that when resources are scarce, individuals may have to allocate resources to either survivorship or reproduction at the expense of the other (Zera & Harshman 2001). This can be adapted to include carotenoids because they are presumed rare in nature and therefore organisms may have to "decide" whether to allocate them to sexual ornamentation or to somatic functions such as immunity or antioxidant activity (Lin et al. 2010). In a study conducted on male zebra finches (*Taeniopygia guttata)*, a species in which the bill is a carotenoid-based ornament, males were supplemented with increasing levels of carotenoids for four weeks and half of the males received weekly doses of *E coli* as an immunological challenge. Carotenoid supplementation increased both circulating carotenoids and the expression of

the ornament. However, in the immune-challenged males, carotenoid levels in circulating plasma and the level of ornamentation decreased (Alonso-Alvarez et al. 2004). Therefore, while there are reproductive advantages to allocating carotenoids to ornamentation, carotenoids also have important somatic functions in terms of immunity and antioxidant activity and the scarcity of carotenoids may require individuals to implement a trade-off in terms of their allocation.

I examined the chemical basis of the red integument ornament, the allocation patterns of carotenoids for different integument types and muscle tissue, the reliability of the red integument ornament in terms of the correlation between carotenoid concentration and spectral properties of the colouration, the life history trade-off of carotenoids allocated to ornamentation versus muscle tissue, and the natural source of carotenoids in male redside dace (*Clinostomus elongatus*). The redside dace is a small cyprinid with a discontinuous distribution that occurs in Southern Ontario in tributaries of Lake Ontario, Lake Erie, and Lake Huron (Parker et al. 1988). The range of the redside dace in the United States includes the headwater streams in the Great Lakes and upper Mississippi River watersheds ranging from Minnesota to New York States (Novinger & Coon 2000). Redside dace have laterally compressed bodies and a black dorsal end with a yellow lateral band directly below. A black band begins at the tip of the snout and runs laterally to the back of the head. A red band occurs laterally along the side and intensifies in colour during the spawning season in males. At the midline, the red band becomes black and ends at the tail. The ventral surface is silver in colour (Parker et al. 1988, See Fig. 2.1). Redside dace are external fertilizers, have a non-resource based mating system, and are sexually dimorphic in colouration with females exhibiting less red colouration than

males (Parker et al. 1988, Beausoleil 2009). Because of this sexual dimorphism, it is likely that the red integument is a sexual ornament in male redside dace (Beausoleil 2009). The coloured integument, particularly the red, is likely carotenoid based and redside dace presumably attain these carotenoids from their diet, which is composed primarily of insects which previous studies have shown may contain carotenoids (Daniels & Wisniewski 1994, Schwartz & Norvel 1958, Matsuno et al. 1999). Redside dace have a large, upturned mouth and are known aerial feeders (Parker et al. 1988). Two populations of redside dace from different drainages in New York State were monitored to determine the components of their diet (Daniels & Wisniewski 1994). Dipteran adults made up between 84-88% of the stomach contents at each site. The majority of dipterans were empidids of the genus *Hilara* which accounted for 60-70% of the total diet by number. Other insect orders found in low numbers were Trichoptera, Ephemeroptera, Odonata, Plecoptera, Homoptera, Hemiptera, Coleoptera, Lepidoptera, and Hymenoptera (Daniels & Wisniewski 1994).

In this study I took absorbance spectroscopy readings to investigate the chemical basis of the red integument ornament as well as to quantify carotenoid concentration and total carotenoid content for the red integument, black integument, silver integument, yellow integument, and muscle. Reflectance spectroscopy readings, which provide details about the spectral properties of colour, were correlated with the carotenoid concentration for the red integument ornament to test the reliable signalling hypothesis. The carotenoid concentrations and carotenoid contents were compared across the red integument, black integument, silver integument, yellow integument and the muscle in order to test the life history trade-off hypothesis in terms of carotenoid allocation.

Finally, insect sampling, identification, and carotenoid extractions were performed in order to determine the source of the carotenoids that produce the red integument ornament in the redside dace diet.

Methods

Fish collection

Thirty-two male redside dace were wild-caught using standard seining techniques during the spawning season from a tributary of the North Branch of the Kokosing River Lake in Knox County, Ohio, USA (N40° 32.787' W082° 39.329') between 12 May and 17 May, 2010. Redside dace sex was confirmed by checking for milt.

Colour analyses

Redside dace were euthanized in a water bath containing an overdose of MS-222. Each male was photographed using a digital camera (Canon PowerShot A570IS) with a ruler in the background of the photograph to provide a scale for later analyses of standard length, body surface area, and surface area of red, black, silver, and yellow integument. Standard length, body surface area (excluding fin area), and surface area of all coloured integument types (red, black, silver, yellow) were determined for the right side of each individual using Image J analysis software (www.rsb.info.nih.gov/ij/) (see Beausoleil 2009). I then doubled the value of these metrics in order to account for the other side.

Measurements of hue, saturation and brightness for the red integument only were obtained for the right side of the body for each of the males using reflectance spectrometry immediately after euthanizing the males (see Pitcher et al. 2009, Fig. 2.2).

The spectral readings were taken using an Ocean Optics reflectance spectrometer (USB 4000), a xenon pulse lamp (PX-2), and a bifurcated fibre-optic probe (R-400-7-UV-VIS). The probe tip was encased in a matte-black rubber cork designed to protect the probe from water and standardize the position of the probe. The light standard used reflected greater than 97% of the relevant wavelengths (Labsphere WS-1). All males were dried using a lint-free delicate task wipe (KimWipes) before reflectance spectroscopy readings were taken to eliminate glare. Reflectance was measured at one standard position on the red integument, immediately behind the operculum because this was the only position on the red integument wide enough to eliminate the effects of other colours. Three readings were obtained for each male that incorporated the average of 20 consecutive measurements using OOIBase 32 software. I used CLR software to translate the readings into spectral properties of hue, saturation, and brightness (Montgomerie 2008). Brightness is calculated as the average reflectance value observed across the whole spectrum (Montgomerie 2006). Hue is defined as 50% of the maximum wavelength observed across the whole spectrum (Montgomerie 2006). Saturation is defined as the difference between maximum and minimum reflectance observed across the whole spectrum, divided by brightness (Montgomerie 2006) (see Beausoleil 2009).

After reflectance readings were taken, all individuals were weighed using a Mettler BB240 balance, wrapped in aluminum foil to reduce light exposure and degradation of carotenoids, and placed in a cooler with ice. After no more than six hours, samples were transferred into a -80°C freezer until carotenoid extractions were performed.

Carotenoid extraction

Redside dace were removed from the -80°C freezer and thawed for five minutes with care taken to minimize light exposure. Red integument, yellow integument, silver integument, and black integument were dissected from each male and different colours of integument were kept separate from each other as well as from muscle and blood. Following the integument dissection, all muscle was dissected from the body. All integument and muscle samples were wrapped separately in aluminum foil and stored at -80°C. The heads, brains, bones, fins, and organs were not used for carotenoids analysis in this study.

Carotenoid extractions for integument were performed using protocols from McGraw et al. (2005) with modifications based on Clotfelter et al. (2007). I recorded the weight (± 0.0001g) of each type of integument for each individual using a Sartorius CP324S balance. For the red integument, only one side was used; for the other colours, however, all available integument was used because the red integument was thought to be much more concentrated in terms of carotenoid content than the other types of integument or muscle samples and to save some red integument for future analysis using HPLC. Each integument sample was submerged in 2ml of methyl tertiary-butyl ether (MTBE) in a 9ml glass tube and homogenized using an IKA-Werke T 45 S8 Blade-type homogenizer controlled by a Tekmar Company Tissumizer Control Module. The homogenized contents were then transferred to another 9ml glass tube and the original tube was rinsed with 1ml of MTBE, which was subsequently transferred to the second tube. Two ml of 1% NH_4OH was then added to each sample, which was then vortexed for 1 min and centrifuged for 5 min at 3000 rpm. This process produced two phases

separated by a lipid layer: a carotenoid layer in the MTBE found on top and a layer of pteridines in NH_4OH found below (see McGraw et al. 2005). The two phases were removed and stored in separate glass tubes for absorbance spectroscopy (see below). Colour in the top MTBE phase confirmed presence of carotenoids in the sample (see McGraw et al. 2005).

Extraction of carotenoids from muscle samples were performed using protocols from Li et al. (2005) with modifications. I allowed muscle to thaw for 5 min prior to extraction. The total mass of all muscle was then determined, a 0.5 g sample was used for extraction, and the rest was returned to the -80°C freezer. The 0.5g sample of muscle was submerged in 1mL of acetone in a 9ml glass screw-cap tube and homogenized as above. The tubes were centrifuged at 3500 rpm for 5 min at 4°C and the supernatant was removed and placed in another 9ml glass screw-cap tube. This procedure was repeated twice and 0.5ml of acetone was added to the original glass tube each time. All supernatants were pooled in a 9ml glass tube. 0.5 ml each of MTBE, hexane, and distilled water were added, and the tube was centrifuged at 3500 rpm for 5 min at 4°C. The organic supernatant on the top layer was removed and put into a separate glass tube. This procedure was repeated twice and supernatants were pooled in this separate glass tube. Pooled supernatants were then evaporated under nitrogen gas and stored in a -80°C freezer for absorbance spectroscopy (see below).

Absorbance spectroscopy

In order to quantify carotenoid content in each of the integument or muscle samples, a Mikropack light-source (DH-2000 UV-VIS-NIR) delivered light through an

Ocean Optics cable to an Ocean Optics 1cm cuvette holder containing a 1.5ml Plastibrand semi-micro UV-cuvette. Light passed through the cuvette into another cable then into an Ocean Optics spectrometer (USB4000 UV-VIS) to generate absorbance spectra. Before processing integument or muscle samples, a blank standard was used. The blank standard was MTBE for carotenoids in integument.

Muscle samples were re-suspended in ethanol in order to determine carotenoid content. Ethanol was used as the blank standard for carotenoids in muscle. Carotenoid concentration for all tissue types was determined using the equation from McGraw et al. (2001): [(absorbance) (volume of extract (ml))] / [(extinction coefficient) (sample mass (g))]. The absorbance was defined as the absorbance of the sample at λmax (See Fig. 2.3, Table 2.3). The extinction coefficient at 1%/1 cm of the relevant carotenoids at λmax was assumed to be 2550, which is consistent with the range of mean maximum absorbance observed in my samples. A subset of red integument samples was examined using HPLC analysis and confirmed the presence of keto-carotenoids (unpublished data). Five readings were obtained for each sample type for each male that incorporated the average of 100 consecutive measurements using OOIBase 32 software.

Insect collection

On 9 May, 2011, four insect traps were placed in an area where redside dace often congregated during the previous spawning season in a tributary of the North Branch of the Kokosing River Lake in Knox County, Ohio, USA (N40° 32.787' W082° 39.329'). Insect traps were constructed by securing an acetate sheet covered in Tree Tanglefoot™ (see Blake & Hoppes 1986) to a nine-inch section of PVC tube (4" diameter) and

mounting that tube on a three-foot wooden stake. The stakes were hammered into the stream bed (see specific locations below) until the plastic tubing was positioned approximately one inch above the water surface where redside dace feed (see Fig. 2.4). On 10 May, 2011, four additional insect traps were placed in an area of overhanging vegetation where redside dace were also known to frequent. On 11 May, 2011 four additional poles were placed in an area where redside dace often fed during the previous spawning season. On May 12, 2011 all insect traps were removed, the acetate sheets were removed from the traps, and were placed in a cooler with ice.

The insects were removed from the acetate sheets and identified to taxonomic order using a key (Marshall 2007). Insects were pooled together by order and the mass was recorded. Carotenoid extraction and absorbance spectroscopy were performed using the same method as was used for the integument (see above, see Fig. 2.12).

Statistical analyses

Data were tested for normality using the Shapiro-Wilk test. If data did not conform to parametric assumptions, I transformed the data using appropriate transformations. I used log transformation on muscle carotenoid content and the silver integument carotenoid content.

A one-way analysis of variance (ANOVA) was performed to determine the differences between the carotenoid concentration and carotenoid content of the red integument, black integument, silver integument, yellow integument, and muscle samples. Post-hoc Tukey tests were performed to determine if and when there were significant differences between the sample types. Pearson correlations were used when

data conformed to parametric statistic assumptions and Spearman correlation were used when data would not conform to parametric assumptions, despite attempts to transform the relevant data. Pearson correlations were performed to determine the relationship between (i) the carotenoid content in the red integument and the carotenoid content combined in the black, silver and yellow integument, (ii) muscle carotenoid content with the total combined integument (red, black, silver, yellow) carotenoid content, (iii) muscle carotenoid content with the red integument carotenoid content, and (iv) muscle carotenoid content with the combined black, silver, and yellow integument carotenoid content. Spearman correlations were performed to determine the relationships between (i) hue with saturation of the red integument, (ii) brightness with saturation of the red integument, (iii) brightness with hue of the red integument, (iv) brightness of the red integument with carotenoid concentration of the red integument, (v) saturation of the red integument with carotenoid concentration of the red integument, and (vi) hue of the red integument with carotenoid concentration of the red integument.

Results

Biological data

Male redside dace standard length had a mean of 79.50 mm, a standard deviation of 5.44 mm, and a range of 71.75 mm to 91.18 mm. Male redside dace body mass had a mean of 4.246 g, a standard deviation of 0.965 g, and a range of 2.955 g to 6.529 g. Individual male redside dace biological data is reported in Table 2.1.

Carotenoid identification

Carotenoids were identified in each sample type (red, black, silver and yellow integument as well as muscle) with λmax (maximum absorbance) values consistent with keto-carotenoids. This was confirmed by performing HPLC analysis on a subset of samples (unpublished data). See Table 2.2 for a descriptive report of the mass, percent of total integument, carotenoid concentration, and carotenoid content for all integument types (red, black, silver and yellow) as well as mass, percent of total body mass, carotenoid concentration, and carotenoid content for muscle.

Allocation

There was significant variation in carotenoid concentration (µg/g) among the different sample types (red, black, silver, and yellow integument as well as muscle); $F = 310.11$, $p < 0.001$, $n = 32$; Fig. 2.5 a). Post-hoc Tukey tests revealed that red integument was significantly different in terms of carotenoid concentration from all samples, black integument was significantly different from all samples, and muscle was significantly different from all samples. Silver integument and yellow integument were significantly different from all samples except each other. There was also significant variation in carotenoid content (µg) among the different samples types (red, black, silver, and yellow integument as well as muscle); $F = 536.25$, $p < 0.001$, $n = 32$; Fig. 2.5 b). Post-hoc Tukey tests revealed that red integument was significantly different from all samples and black integument was significantly different from all samples. Silver integument, yellow integument, and muscle were significantly different from all samples except each other.

Figure 2.6 displays the allocation of carotenoid content converted to a percentage of the total carotenoid content throughout the different sample types per individual (n =

32). Red integument had the most carotenoids followed by black integument, muscle, yellow integument and silver integument. Red integument carotenoid content was a mean of 70.8% of the total carotenoid availability with a range of 61.1% to 77.6% and a standard deviation of 4.2%. Black integument carotenoid content was a mean of 18.6% of the total carotenoid availability with a range of 13.3% to 24.1% and a standard deviation of 2.9%. Silver integument carotenoid content was a mean of 2.7% of the total carotenoid availability with a range of 1.3% to 5.5% and a standard deviation of 1.0%. Yellow integument carotenoid content was a mean of 3.4% of the total carotenoid availability with a range of 1.5% to 6.0% and a standard deviation of 1.2%. Muscle carotenoid content was a mean of 4.6% of the total carotenoid availability with a range of 2.0% to 11.6% and a standard deviation of 2.4%.

Life- History Trade-off hypothesis

There was a significant positive relationship between red integument carotenoid content and the combined carotenoid content of black, silver, and yellow integuments ($r = 0.66$, $p < 0.001$, $n = 32$; Fig. 2.7). There was a significant relationship between red integument carotenoid content and black integument carotenoid content ($r = 0.62$, $p < 0.001$, $n = 32$), silver integument carotenoid content ($r = 0.65$, $p < 0.001$, $n = 32$), and yellow integument carotenoid content ($r = 0.40$, $p = 0.03$, $n = 32$).

There was a significant relationship between muscle carotenoid content and combined integument carotenoid content ($r = 0.39$, $p = 0.03$, $n = 32$; Fig. 2.8 a) and between muscle carotenoid content and black, silver, and yellow integument carotenoid content combined ($r = 0.43$, $p = 0.01$, $n = 32$; Fig. 2.8 b). There was a nearly significant

relationship between muscle carotenoid content and red integument carotenoid content ($r = 0.36$, $p = 0.06$, $n = 32$; Fig 2.8 c).

Spectral properties

For the red integument, there was a significant negative relationship between brightness and saturation ($r_s = -0.47$, $p = 0.01$, $n = 32$; Fig. 2.9 a), but no significant relationship existed between hue and saturation ($r_s = 0.26$, $p = 0.15$, $n = 32$; Fig. 2.9 b) or brightness and hue ($r_s = -0.08$, $p = 0.67$, $n = 32$; Fig. 2.9c).

Reliable signalling hypothesis

There was a marginally significant negative relationship between spectral measures of saturation and carotenoid concentration in red integument ($r_s = -0.35$, $p = 0.05$, $n = 32$; Fig. 2.10a). There was no significant relationship between spectral measures of brightness ($r_s = 0.23$, $p = 0.21$, $n = 32$; Fig. 2.10b) and hue ($r_s = -0.15$, $p = 0.41$, $n = 32$; Fig. 2.10c) of red integument in relation to carotenoid concentration.

Insects

The insect sampling protocol resulted in the collection of a variety of insects (n = 461 individuals), the majority originating from the order Diptera (n = 340) and Coleoptera (n = 70) (see Table 2.4). The total mass of all of the Dipterans was 0.7114g, making the mean mass per individual 0.00209g (0.7114g / 340 individuals). The total mass of all of the Coleopterans was 0.1444g, making the mean mass per individual 0.00206g (0.1444g / 70 individuals). The carotenoid concentration was 2.99 ug/g and

5.37 ug/g for Dipterans and Coleopterans, respectively. Therefore, the carotenoid content of each Dipteran and Coleopteran was $6.25x10^{-3}$ ug and 0.01 ug, respectively (see Table 2.4).

Discussion

My findings suggest that keto-carotenoids are the chemical basis of the red integument in redside dace. This is supported by preliminary HPLC analysis on a subset of samples (unpublished data). Carotenoids were present throughout the tissue types of the body and the majority of carotenoids were allocated to the red integument. Additionally, there was evidence that males with more carotenoids in their red integument also had more carotenoids to allocate to their other tissue types. I found no evidence that a trade-off occurs between carotenoids in the red integument and muscle tissue and no evidence that the spectral properties of the red integument are a reliable signal of carotenoid concentration.

Redside dace allocated carotenoids throughout their integument and muscle tissue in a similar pattern with slight variation between individuals. However, the majority was allocated to the red integument. This was predicted because visually the red integument is relatively large with a lot of colour and also because these redside dace were sampled during their spawning season where allocation to colouration for sexual ornamentation or social signalling between males should be at its highest (Garner et al. 2010, Olsson 1994). This phenomenon is observed in other organisms such as zebra finches (*Taeniopygia guttata)*. In zebra finches, the colouration is on the integument of the legs and beak. Carotenoid levels were tested for the liver, adipose tissue, retina, integument

on the legs, and integument on the beak. The integument of the beak had the highest levels of carotenoid concentration, followed by the integument of the leg, the adipose tissue, the retina, and finally the liver (McGraw & Toomey 2010). This is consistent with the results of my study as the majority of carotenoids are allocated to colouration.

The life history trade-off hypothesis predicts that in times of scarcity, resources may have to be allocated to either reproduction or survival at the expense of the other (Zera & Harshman 2001). In this study, the resource in question was carotenoids as they may be allocated to colouration or somatic physiological functions (Lin et al. 2010, Britton 2008). Carotenoids are scarce in this system because the primary dietary source for redside dace is terrestrial insects and I found that the average dipteran contains $6.25x10^{-3}$ µg of carotenoids, the average coleopteran contains 0.01 µg of carotenoids, and on average, the redside dace I analyzed contained 25.03 µg of carotenoids in the integument and muscle tissue. I found no evidence to support the trade-off hypothesis because that would have required a negative correlation between somatic carotenoids and colouration carotenoids. There is evidence in the literature for the occurrence of a life-history trade-off hypothesis with carotenoids. Studies conducted on captive birds have shown that following an immunological challenge, carotenoids are diverted from colouration and allocated to circulating plasma (see Alonso-Alvarez et al. 2004, Alonso-Alvarez et. al 2008). Additionally, a study conducted on wild-caught three-spined sticklebacks, showed that males fed low carotenoid diets were significantly more susceptible to oxidative stress than males fed high carotenoid diets (Pike et al. 2007). However, this evidence differs from my study because I did not experimentally manipulate diet or immune response and I used muscle tissue to represent somatic

carotenoids as opposed to plasma. Additionally, it is possible that I did not find evidence to support this hypothesis because the life-history trade-off is occurring using one specific type of carotenoid (e.g. astaxanthin) as opposed to my metric of total carotenoid content. In a study conducted on house finches, different carotenoids were found in liver and plasma while β-cryptoxanthin was found to be the carotenoid responsible for colouration (McGraw et al. 2006). A pattern such as this is capable of clouding the trade-off results in my study because it is possible that only one carotenoid is utilized in terms of colouration in redside dace as well.

Although I did not observe a relationship consistent with a trade-off, I did see evidence that greater carotenoid content in the red integument was indicative of greater overall carotenoid content in the body and in the different tissue types individually. This may occur because some males have elevated foraging skills or because of an individual's physiological ability to only possess a finite amount of carotenoids (Searcy & Nowicki 2005, McGraw et al. 2002). Evidence for a similar pattern has been observed in zebra finches. It was found that males with more brightly coloured bills that presumably had more carotenoids deposited, had higher levels of circulating carotenoids in the plasma (McGraw et al. 2003). These results differ from mine because this study involved dietary manipulations however, the same pattern of males with more carotenoids in their colouration having more to allocate throughout their other tissue was observed.

I did not find evidence to support the reliable signalling hypothesis in terms of a consistent positive correlation between any of the spectral properties of the red integument and carotenoid concentration of the red integument. However, I did observe

a marginally significant negative correlation between saturation and carotenoid concentration of the red integument which may suggest deceptive signalling. I may not have observed the pattern that I expected because of my methodology for determining carotenoid concentration, in which only total carotenoids and not individual constituent carotenoids are taken into account (Pike et al. 2011). Studies of sticklebacks and house finches have shown that spectral properties correlated with individual carotenoids only and not total carotenoid content (Wedekind et al 1998, Inouye et al. 2001). Additionally, it is possible that the red integument is a deceptive signal in terms of the spectral properties.

It is known that carotenoids are not the only molecules available for colouration. Pteridines are also used in carotenoid-based colouration. Pteridines are purine derivatives that organisms are able to produce de novo by synthesizing carbohydrates and proteins (Grether et al. 2001). For example, in guppies (*Poecilia reticulata*), the orange spots on the integument were analyzed to determine if the colouration was an honest carotenoid-based signal or if guppies were supplementing their colouration with pteridines (Grether et al. 2001). It was found that both carotenoids and pteridines were present in guppies but they were highly correlated with each other. Therefore male guppies are not using pteridines to make up for a lack of carotenoids and the orange spots on guppies are indeed honest signals of their carotenoid content (Grether et al. 2001). In a post-hoc analysis of my dataset, I found that there was a significant positive relationship between pteridine concentration in the red integument and carotenoid concentration in the red integument ($r_s = 0.51$, $p = 0.003$, $n = 32$; Fig. 2.11), suggesting that redside dace are not

using pteridines to supplement their red integument colouration as males with low levels of carotenoids also have low levels of pteridines and vice versa.

Due to the sexual dimorphism and intensity of colouration of the red integument, I tested the reliable signalling hypothesis with the assumption that males were signalling their carotenoid content to females in terms of the visual spectral properties of their red integument for the purpose of female mate choice. However, new research indicates that female redside dace show no significant difference in preference for males with different spectral properties in their red integument based on the results of mate choice trials. This suggests that the red integument of the male redside dace is not a sexual ornament and therefore, may serve some other function such as social status signalling (Beausoleil 2009).

There are few studies that investigate the source of carotenoids in a wild population that displays carotenoid-based colouration (Matsuno et al. 1999). In this study, I performed the preliminary work to determine that Diptera are the most abundant insects and Coleoptera are also available in the feeding area of redside dace which is consistent with previous research (Daniels & Wisniewski 1994, Schwartz & Norvel 1958). I determined that Diptera and Coleoptera contain carotenoids and therefore are a dietary source of carotenoids for redside dace which is consistent with the results of previous research performed on Chinese minnows (*Rhynchocypris oxycephalus)* (Matsuno et al. 1999). The fact that carotenoids are presumably obtained from terrestrial insects has implications for this study. It may be difficult for redside dace to catch these insects which contain small amounts of carotenoids relative to redside dace. This provides evidence that carotenoids are rare or that obtaining a lot of carotenoids is an

indication of elevated foraging skills which helps to justify testing the reliable signalling hypothesis with respect to carotenoids in redside dace (Searcy & Nowicki 2005). Also, the difficulty involved in obtaining carotenoids could be further support for the need for a trade-off.

This study provided evidence that the red integument in redside dace is keto-carotenoid based, that redside dace are obtaining carotenoids through their terrestrial insect diet, and that male redside dace with more carotenoids to allocate to colouration have more carotenoids to allocate throughout the rest of their body. This study did not provide evidence supporting the reliable signalling or life-history trade-off hypotheses. This was the first study to confirm that the red integument in redside dace is carotenoid-based. Additionally, this study provided descriptive information about carotenoid allocation and spectral properties of red integument in a wild population of redside dace. Research has shown that lab-based signalling studies based on colouration may not be accurate in terms of the spectral properties obtained from captive animals (Barber et al. 2000). This is also one of few studies to identify the natural source of carotenoids and in doing so, demonstrates the rarity of carotenoids in the environment of a wild population of fish that display carotenoid-based colouration. The next steps to be taken to further understand reliable signalling in redside dace are to incorporate a visual model to ensure spectral properties accurately represent redside dace vision, perform HPLC analysis to determine constituent carotenoids and how they may compare with spectral properties, and further investigate redside dace carotenoid colouration and life-history trade-offs by manipulating diet and immune response (Pike et al. 2011, Wedekind et al. 1998, Alonso-Alvarez et al. 2004).

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Table 2.1 Mean, standard deviation, and range for the standard length (mm) and the body mass (g) for each individual redside dace (*Clinostomus elongatus*) (n = 32).

Standard Length (mm)	Body Mass (g)
80.28	4.856
82.17	5.099
73.35	3.474
83.33	4.973
72.36	3.122
91.01	6.529
75.78	3.733
84.49	5.348
83.81	4.875
88.74	6.294
78.53	4.372
78.35	3.884
91.18	5.762
78.76	4.138
74.99	3.551
87.41	5.570
80.36	3.794
75.74	3.342
79.91	3.998
75.29	3.312
73.61	3.043
76.4	3.704
83.76	4.620
86.22	5.277
73.85	3.482
71.75	3.193
80.06	4.088
72.93	3.460
75.66	3.674
80.55	4.464
76.17	3.887
77.17	2.955

Table 2.2 Mean, standard deviation, and range for the mass (g), percent mass of total integument (%), carotenoid concentration (µg/g), and carotenoid content (µg) for the different integument types (red, black, silver and yellow) and mean, standard deviation, and range for the mass (g), percent mass of total body mass (%), carotenoid concentration (µg/g), and carotenoid content for muscle (µg) averaged for each individual redside dace (*Clinostomus elongatus*) (n = 32).

Tissue type	Metric	Mean	Standard Deviation	Range
Red Integument	Mass (g)	0.0636	0.0244	0.0292 - 0.1104
	Mass (% integument)	29.13	5.72	20.66 – 40.46
	Carotenoid Concentration (µg/g)	297.45	86.75	174.88 - 516.52
	Carotenoid Content (µg)	17.70	3.66	10.25 - 25.01
Black Integument	Mass (g)	0.0801	0.0283	0.0377-0.1748
	Mass (% integument)	36.70	5.13	26.00 – 45.58
	Carotenoid Concentration (µg/g)	59.61	11.48	34.01 - 82.87
	Carotenoid Content (µg)	4.64	1.15	2.51 - 6.72
Silver Integument	Mass (g)	0.0376	0.0169	0.0098 - 0.0956
	Mass (% integument)	16.74	5.21	9.30 – 26.87
	Carotenoid Concentration (µg/g)	20.26	9.66	6.33 - 49.43
	Carotenoid Content (µg)	0.69	0.34	0.26 - 1.57
Yellow Integument	Mass (g)	0.0373	0.0099	0.0210 - 0.0604
	Mass (% integument)	17.42	3.49	9.10 – 26.43
	Carotenoid Concentration (µg/g)	22.89	9.31	11.30 - 57.96
	Carotenoid Content (µg)	0.84	0.33	0.35 - 1.78
Muscle	Mass (g)	1.1462	0.3319	0.7179 – 1.9985

	Mass (% body mass)	26.86	3.41	19.19 – 32.24
	Carotenoid Concentration (µg/g)	1.06	0.61	0.37 – 2.86
	Carotenoid Content (µg)	1.16	0.64	0.35 – 3.23

Table 2.3 Light absorbance between wavelengths of 300 nm – 700 nm averaged for each individual redside dace (*Clinostomus elongatus*) (n = 32) over five readings per individual and averaged per sample type. The table shows the mean (+/- S.E.) reading for each 10 nm.

Wavelength (nm)	Red integument absorbance		Black integument absorbance		Silver integument absorbance		Yellow integument absorbance		Muscle absorbance	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
300-309	0.239	0.052	0.004	0.002	0.000	0.000	0.002	0.001	0.052	0.013
309-319	0.230	0.050	0.005	0.003	0.000	0.000	0.002	0.001	0.069	0.015
319-329	0.232	0.050	0.007	0.004	0.000	0.000	0.003	0.001	0.076	0.016
329-339	0.215	0.048	0.005	0.003	0.000	0.000	0.001	0.000	0.054	0.013
339-349	0.228	0.049	0.006	0.003	0.000	0.000	0.001	0.001	0.039	0.010
349-359	0.247	0.051	0.004	0.002	0.000	0.000	0.001	0.001	0.018	0.007
359-369	0.319	0.057	0.011	0.006	0.000	0.000	0.005	0.003	0.012	0.005
369-379	0.423	0.062	0.034	0.012	$1.5X10^{-6}$	$1.5X10^{-6}$	0.010	0.006	0.013	0.006
379-389	0.589	0.064	0.144	0.020	0.002	0.001	0.027	0.010	0.023	0.007
389-399	0.750	0.067	0.299	0.022	0.011	0.004	0.061	0.012	0.037	0.009
399-409	0.932	0.071	0.449	0.025	0.034	0.007	0.103	0.013	0.053	0.010
409-419	1.152	0.071	0.646	0.029	0.088	0.011	0.155	0.013	0.073	0.012
419-429	1.348	0.073	0.803	0.032	0.129	0.013	0.185	0.014	0.085	0.011
429-439	1.569	0.070	0.971	0.039	0.157	0.014	0.191	0.014	0.084	0.010
439-449	1.732	0.069	1.113	0.044	0.174	0.015	0.208	0.014	0.083	0.009
449-459	1.812	0.069	1.123	0.048	0.160	0.014	0.184	0.013	0.078	0.009
459-469	1.956	0.069	1.058	0.061	0.128	0.011	0.143	0.010	0.061	0.007
469-479	2.068	0.078	0.972	0.069	0.113	0.009	0.131	0.009	0.052	0.005
479-489	2.057	0.095	0.676	0.051	0.083	0.007	0.092	0.007	0.042	0.004
489-499	1.903	0.112	0.377	0.025	0.054	0.005	0.046	0.005	0.029	0.003
499-509	1.596	0.132	0.214	0.014	0.036	0.004	0.022	0.004	0.020	0.003
509-519	1.160	0.133	0.132	0.009	0.026	0.003	0.013	0.003	0.015	0.003
519-529	0.672	0.084	0.083	0.006	0.020	0.002	0.008	0.003	0.013	0.003
529-539	0.317	0.031	0.051	0.004	0.015	0.002	0.006	0.002	0.011	0.003
539-549	0.151	0.014	0.033	0.004	0.013	0.002	0.004	0.002	0.011	0.003

549-559	0.078	0.008	0.025	0.004	0.013	0.002	0.004	0.002	0.010	0.003
559-569	0.049	0.006	0.021	0.004	0.012	0.002	0.004	0.002	0.009	0.003
569-579	0.037	0.005	0.019	0.004	0.010	0.002	0.004	0.002	0.009	0.003
579-589	0.032	0.005	0.017	0.003	0.011	0.002	0.003	0.002	0.009	0.003
589-599	0.029	0.005	0.017	0.003	0.011	0.002	0.003	0.002	0.009	0.003
599-609	0.027	0.005	0.017	0.003	0.011	0.002	0.003	0.002	0.009	0.003
609-619	0.025	0.005	0.016	0.003	0.010	0.002	0.003	0.002	0.009	0.003
619-629	0.024	0.004	0.015	0.003	0.010	0.002	0.003	0.002	0.009	0.004
629-639	0.023	0.004	0.015	0.003	0.009	0.002	0.003	0.002	0.009	0.004
639-649	0.023	0.004	0.015	0.003	0.010	0.002	0.003	0.002	0.009	0.004
649-659	0.023	0.004	0.015	0.003	0.009	0.002	0.003	0.002	0.009	0.004
659-669	0.022	0.004	0.015	0.003	0.009	0.002	0.003	0.002	0.009	0.004
669-679	0.021	0.004	0.013	0.003	0.009	0.002	0.003	0.001	0.009	0.004
679-689	0.021	0.004	0.013	0.003	0.009	0.002	0.002	0.001	0.009	0.004
689-699	0.020	0.004	0.013	0.003	0.008	0.002	0.003	0.002	0.009	0.004

Table 2.4 Total number, relative abundance, total combined mass (g), mass per insect (g), carotenoid concentration (µg/g), and carotenoid content per insect (µg) for insects collected from five Orders (Diptera, Coleoptera, Lepidoptera, Hymenoptera, and Odonata) as well as Arachnids and unknown (n = 461). Carotenoid analysis was conducted only on Diptera and Coleoptera because of the mass needed for analysis.

Order	Total number	Relative abundance (%)	Total combined mass (g)	Mass per insect (g)	Carotenoid concentration (µg/g)	Carotenoid content (µg)
Diptera	340	73.75	0.7114	0.0002	2.99	$6.25X10^{-3}$
Coleoptera	70	15.18	0.1444	0.0002	5.37	0.01
Lepidoptera	12	2.60				
Arachnidae	9	1.95				
Hymenoptera	1	0.22				
Odonata	1	0.22				
Other	28	6.07				

Fig. 2.1

Fig. 2.1 Typical male redside dace (*Clinostomus elongatus*) during the spawning season with black circle indicating landmark position for reflectance spectrometry (see Methods) and a scale bar representing 5mm (adapted from Beausoleil 2009).

Fig 2.2

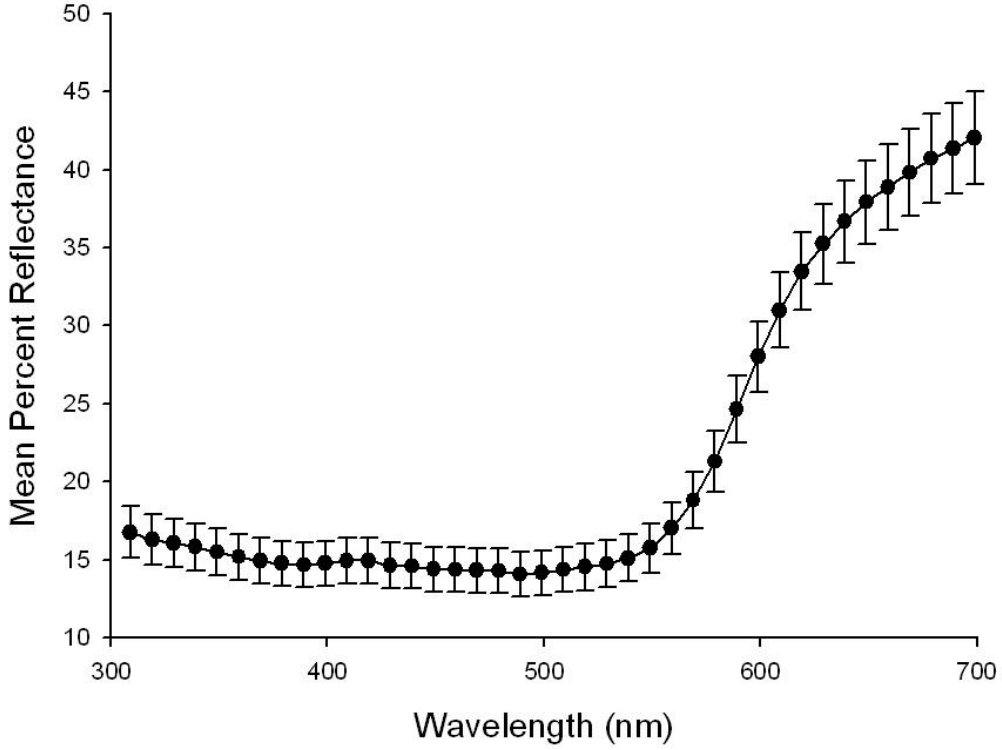
Mean Percent Reflectance
50
45
40
35
30
25
20
15
10
300
400
500
600
700
Wavelength (nm)

Figure 2.2 The percent reflectance between wavelengths of 300 nm – 700 nm for the red integument ornament of each individual redside dace (*Clinostomus elongatus*) over three readings per individual and averaged over all individuals. The plot shows the mean (+/- S.E.) reading for each 10 nm of wavelength.

Fig. 2.3

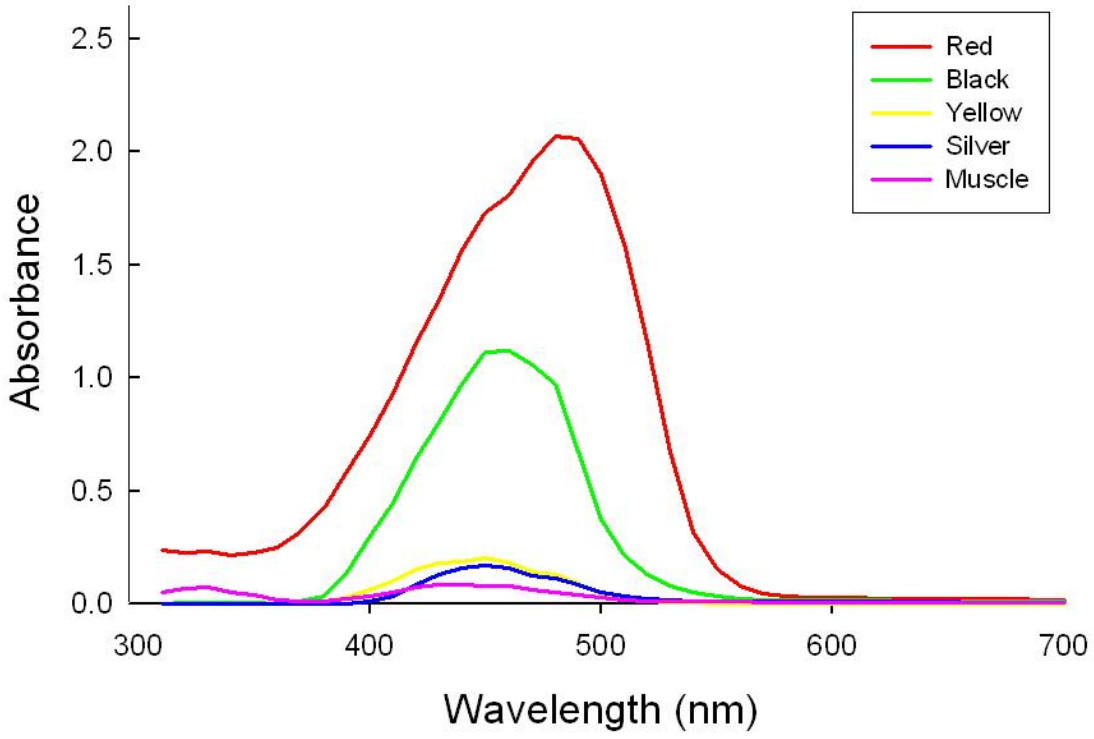
Red
Black
Yellow
Silver
Muscle
Absorbance
2.5
2.0
1.5
1.0
0.5
0.0
300
400
500
600
700
Wavelength (nm)

Fig. 2.3 The absorbance between wavelengths of 300 nm – 700 nm averaged for each individual redside dace (*Clinostomus elongatus*) (n = 32) over five readings per individual and averaged per sample type. The plot shows the mean reading for each 10 nm of wavelength and the standard error is not shown for the sake of clarity. Red integument is represented by a red line, black integument is represented by a green line, silver integument is represented by a yellow line, yellow integument is represented by a blue line, and muscle is represented by a purple line.

Fig. 2.4

Fig. 2.4 Insect trap consisting of an acetate sheet covered in Tree Tanglefoot™ screwed onto a nine-inch section of PVC tube (4" diameter) and mounted on a three-foot wooden stake. Stakes were hammered into the stream bed until the tubing was approximately one inch above the water surface.

Fig 2.5

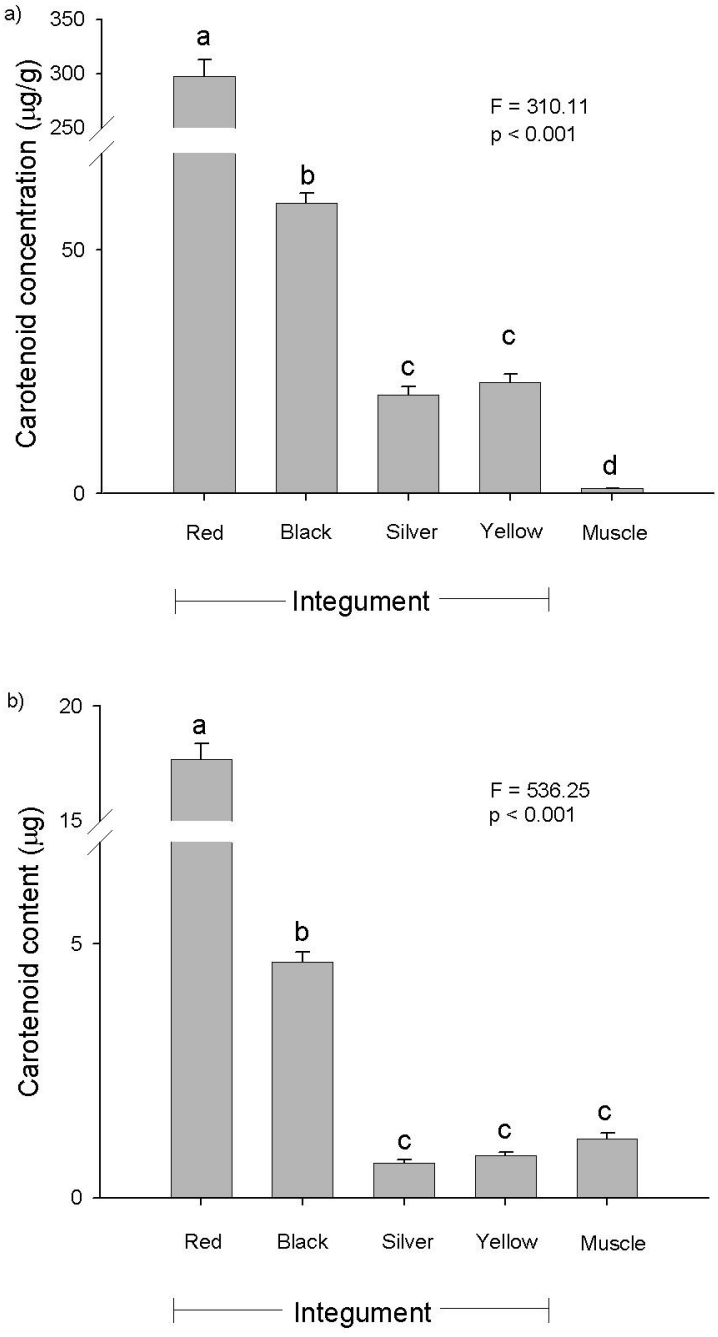
a)
350
300
250
50
0
Carotenoid concentration (µg/g)
a
b
c
c
d
F = 310.11
p < 0.001
Red
Black
Silver
Yellow
Muscle
Integument
b)
20
15
5
0
Carotenoid content (µg)
a
b
c
c
c
F = 536.25
p < 0.001
Red
Black
Silver
Yellow
Muscle
Integument

Fig. 2.5 (a) Mean (+/- S.E.) carotenoid concentration (measured in µg/g) across all individual redside dace (*Clinostomus elongatus*) (n = 32) for the different sample types (red integument, black integument, silver integument, yellow integument, muscle). The letters above the bars signify the results of post-hoc Tukey tests; a different letter signifies a significant difference. (b) Mean (+/- S.E.) carotenoid content (measured in µg) across all individuals (n = 32) for the different sample types (red integument, black integument, silver integument, yellow integument, muscle). The letters above the bars signify the results of a post-hoc Tukey test, a different letter signifies a significant difference.

Fig 2.6

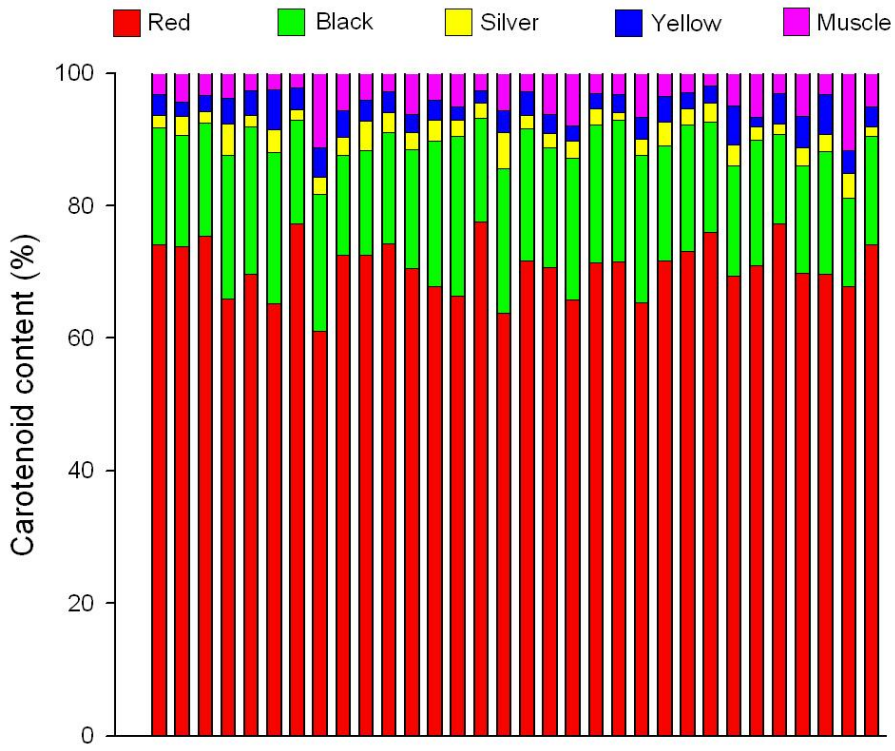
Red
Black
Silver
Yellow
Muscle
Carotenoid content (%)
100
80
60
40
20
0

Fig. 2.6 Carotenoid content allocation per individual redside dace (*Clinostomus elongatus*) (n = 32) converted to a percentage of the total carotenoid content of the individual for the different sample types. Red integument is represented by red bars, black integument is represented by green bars, silver integument is represented by yellow bars, yellow integument is represented by blue bars, and muscle is represented by purple bars.

Fig. 2.7

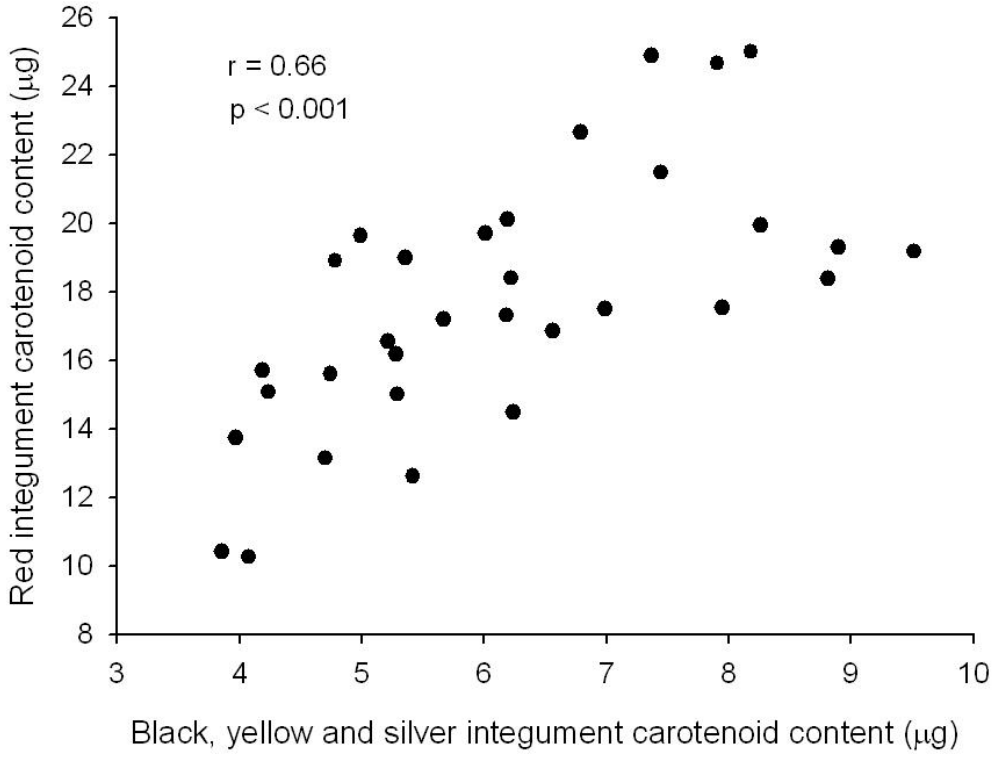
r = 0.66
p < 0.001
Red integument carotenoid content (μg)
Black, yellow and silver integument carotenoid content (μg)
26
24
22
20
18
16
14
12
10
8
3
4
5
6
7
8
9
10

Fig. 2.7 The relationship between red integument carotenoid content and carotenoid content of the black integument, silver integument and yellow integument combined for individual redside dace (*Clinostomus elongatus*) (n=32).

Fig. 2.8

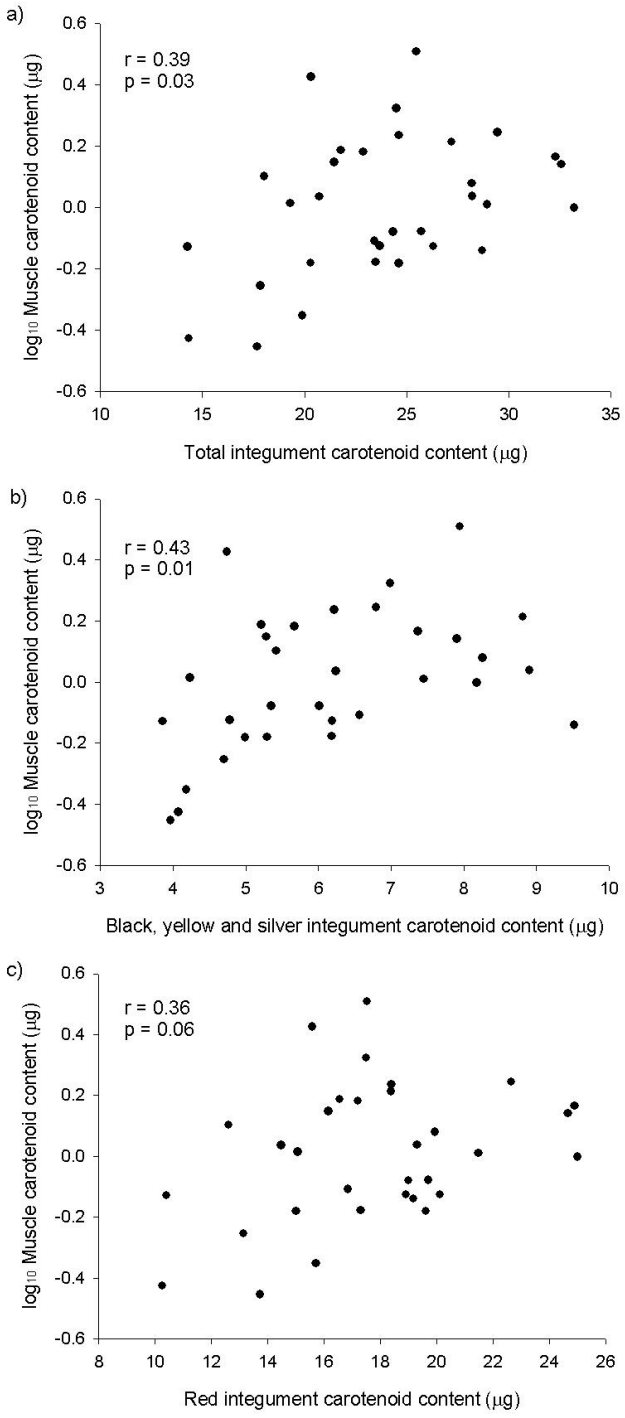
a)
r = 0.39
p = 0.03
log10 Muscle carotenoid content (μg)
Total integument carotenoid content (μg)
b)
r = 0.43
p = 0.01
log10 Muscle carotenoid content (μg)
Black, yellow and silver integument carotenoid content (μg)
c)
r = 0.36
p = 0.06
log10 Muscle carotenoid content (μg)
Red integument carotenoid content (μg)

Fig. 2.8 (a) The relationship between carotenoid content in the muscle and carotenoid content in all of the integument samples combined (red, black, silver, yellow) for individual redside dace (*Clinostomus elongatus*) (n = 32). (b) The relationship between carotenoid content in the muscle and carotenoid content of the black integument, silver integument and yellow integument combined for individual redside dace (n = 32). (c) The relationship between carotenoid content in the muscle and the carotenoid content in the red integument for individual redside dace (n = 32).

Fig. 2.9

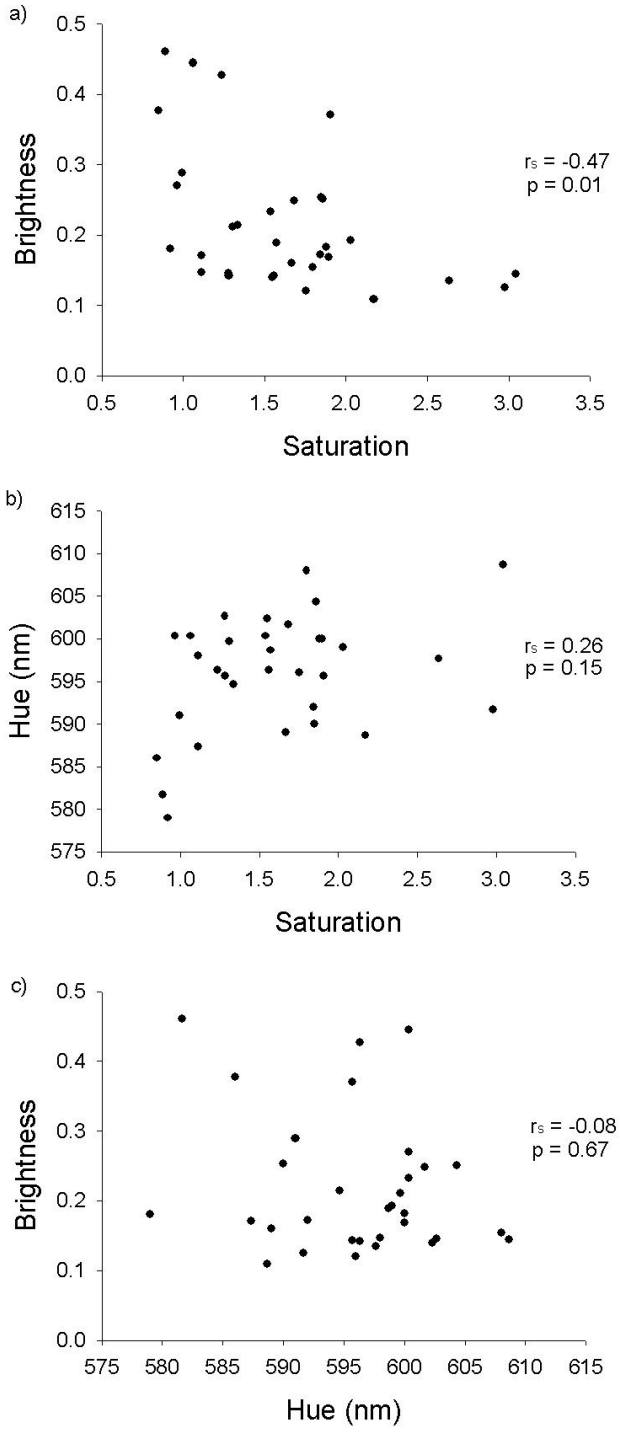
a)
Brightness
Saturation
r_s = -0.47
p = 0.01
b)
Hue (nm)
Saturation
r_s = 0.26
p = 0.15
c)
Brightness
Hue (nm)
r_s = -0.08
p = 0.67

Fig. 2.9 (a) The relationship between the spectral properties brightness and saturation for the red integument ornament for individual redside dace (*Clinostomus elongatus*) (n = 32). (b) The relationship between the spectral properties hue and saturation for the red integument ornament for individual redside dace (n = 32). (c) The relationship between the spectral properties brightness and hue for the red integument ornament for individual redside dace.

Fig. 2.10

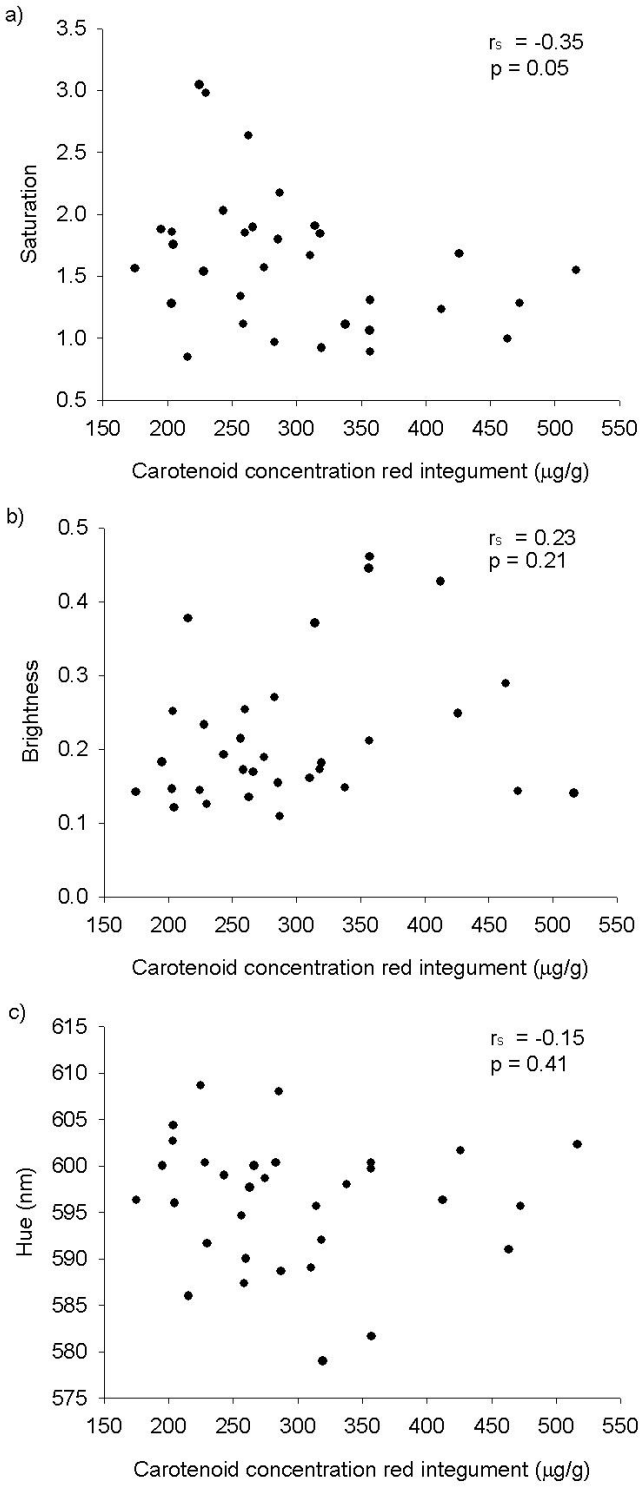
a)
r_S = -0.35
p = 0.05
Saturation
3.5
3.0
2.5
2.0
1.5
1.0
0.5
150 200 250 300 350 400 450 500 550
Carotenoid concentration red integument (µg/g)
b)
r_S = 0.23
p = 0.21
Brightness
0.5
0.4
0.3
0.2
0.1
0.0
150 200 250 300 350 400 450 500 550
Carotenoid concentration red integument (µg/g)
c)
r_S = -0.15
p = 0.41
Hue (nm)
615
610
605
600
595
590
585
580
575
150 200 250 300 350 400 450 500 550
Carotenoid concentration red integument (µg/g)

Fig. 2.10 (a) The relationship between the spectral property saturation and the carotenoid concentration of the red integument ornament for individual redside dace (*Clinostomus elongatus*) (n = 32). (b) The relationship between the spectral property brightness and the carotenoid concentration of the red integument ornament for individual redside dace (n = 32). (c) The relationship between the spectral property hue and the carotenoid concentration of the red integument ornament for individual redside dace (n = 32).

Fig. 2.11

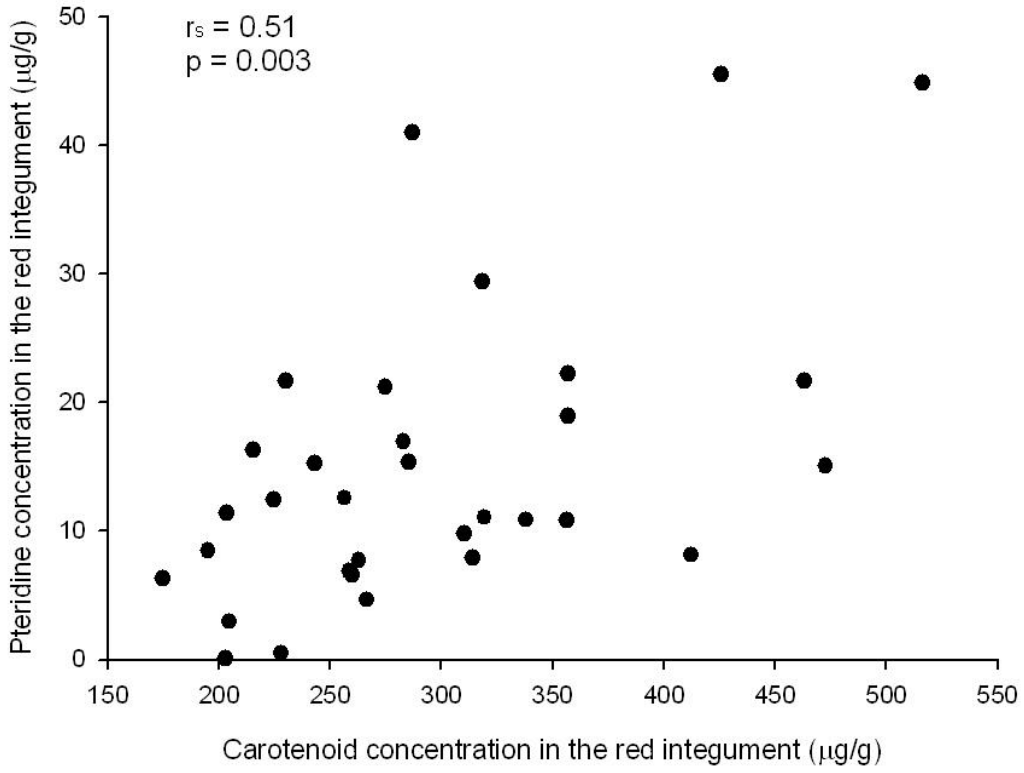
rs = 0.51
p = 0.003
Pteridine concentration in the red integument (μg/g)
Carotenoid concentration in the red integument (μg/g)
0
10
20
30
40
50
150
200
250
300
350
400
450
500
550

Fig. 2.11 The relationship between carotenoid concentration and pteridine concentration of the red integument ornament for individual redside dace (*Clinostomus elongatus*) (n = 32).

Fig. 2.12

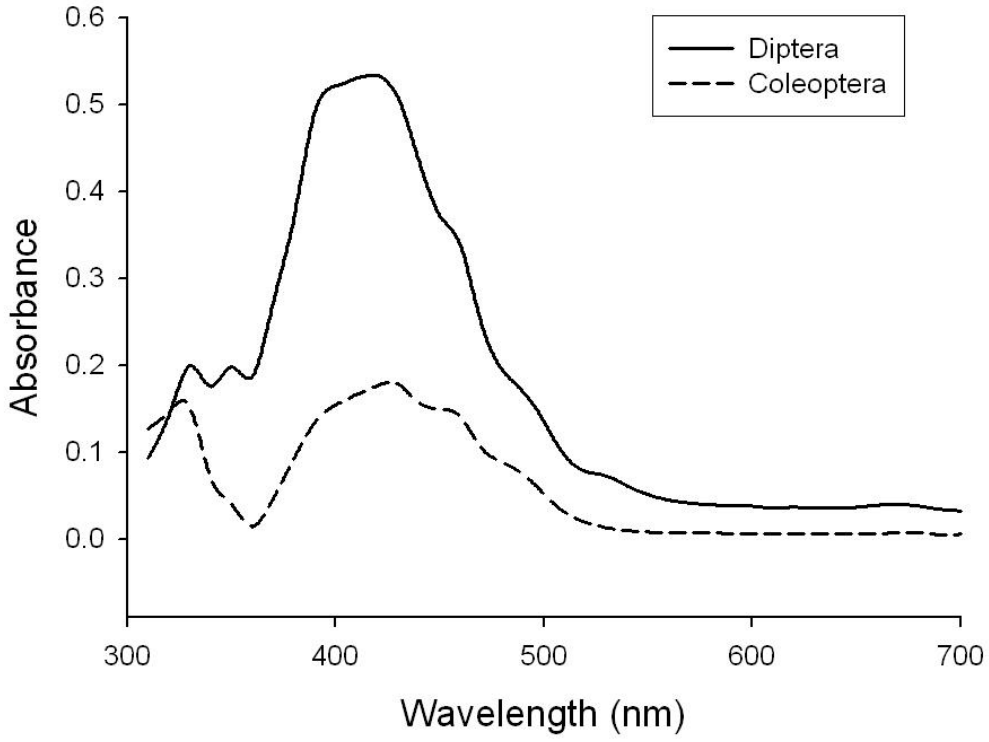
Diptera
Coleoptera
Absorbance
0.6
0.5
0.4
0.3
0.2
0.1
0.0
300
400
500
600
700
Wavelength (nm)

Fig. 2.12 The absorbance between wavelengths of 300 nm – 700 nm averaged for each Order of insects (Diptera and Coleoptera) over five readings. The plot shows the mean reading for each 10 nm of wavelength. Diptera is represented by a solid black line and Coleoptera is represented by a disconnected black line.

CHAPTER 3: GENERAL DISCUSSION

Summary

My research investigated the chemical basis and source of the red integument, carotenoid allocation, life-history trade-off, and reliable signalling hypotheses using the redside dace as a study system. This chapter will serve as a summary of my research and a place to address questions and future directions pertaining to this research.

Chapter Two

A common basis of colouration across taxa is carotenoid deposits on the feathers, integument, or other tissue that causes yellow, orange, or red colouration (Olson & Owens 1998). I confirmed that the chemical basis of the red integument of the redside dace is keto-carotenoids. Furthermore, in studying the allocation of carotenoids to the red integument versus the black integument, silver integument, yellow integument, and muscle tissue, it was found that redside dace allocated the majority of their total body carotenoids to the red integument. Allocating the majority of carotenoids to colouration has also been documented in other species including zebra finches (McGraw & Toomey 2010). I also found no consistent correlation between spectral properties of red colouration and carotenoid concentration for the red integument and I was therefore, unable to refer to the red integument of the redside dace as a reliable signal. In terms of a life history trade-off, there was no evidence that the redside dace were performing any trade-off between the somatic carotenoids and the carotenoids allocated to the red integument.

Reliable signalling hypothesis

I did not observe a positive correlation between spectral properties and carotenoid concentration of the red integument in redside dace, which I determined to be an important attribute using evidence from the literature (Searcy & Nowicki 2005, Olson & Owens 1998, Smith & Harper 2003). Therefore, my data does not presently provide evidence to support the idea that the red integument is a reliable signal in terms of spectral properties and carotenoid concentration for this species. In order to explore the reason for this, it is important to understand spectral properties and carotenoid concentration as metrics. The spectral properties must be representative of what redside dace are actually able to see in order to be used to test the reliable signalling hypothesis. Although many studies in the past have used subjective human rankings to test colour, I used reflectance spectrometry between the wavelengths of 300 nm and 700 nm in order to remove human bias (Bennett et al. 1994). In terms of vision in the redside dace, their environment must be taken into consideration. While many fishes have limited sight due to water depth or salt content in the water, redside dace live near the surface in fresh water and therefore the majority of photons of light will be able to reach their optical system (Levine & MacNichol 1982). Again, due to the varying environments of fishes, different species have different capabilities in terms of vision (Levine & MacNichol 1982). However, fishes that live in environments with a lot of light have developed multiple cones and rods capable of colour and ultraviolet vision (Levine & MacNichol 1982). Although there has been no research of the vision of redside dace to my knowledge, goldfish have been extensively studied and they are in the same family as redside dace. Goldfish have been found to have wavelength discrimination between 400

nm - 719 nm and peak sensitivity at three landmark wavelengths: 400 nm - 410 nm, 500 nm, and 600 nm – 610 nm representing violet, blue-green, and orange-red colour vision similar to humans and additionally, ultraviolet vision (Neumeyer 1985). However, redside dace may have different wavelength peak sensitivities as this has been observed in other cyprinids (Neumeyer 1985). Cyprinids have been extensively studied, however, and colour vision has been observed in others including zebra fish (Danio aequipinnatus) and roach (Rutilus rutilus) (Palacios et al. 1996, Downing et al. 1986). Due to redside habitat and feeding method, it is likely that they have colour vision that can be accurately assessed using the method of reflectance spectroscopy that I used for this study. In terms of the carotenoid concentration, I used carotenoid specific extraction methods then absorbance spectroscopy to get an indication of total carotenoid concentration and content in red integument. This may have masked a correlation that could have been observed between one or more individual carotenoid constituents and spectral properties. For example, in a study conducted on sticklebacks, it was found that astaxanthin concentration correlated with red colouration only and tunaxanthin/lutein concentration correlated with yellow colouration only (Wedekind et al. 1998). This evidence leads me to believe that before dismissing the spectral properties of the red integument as a reliable signal in terms of the carotenoid concentration, I should more thoroughly examine the individual carotenoids present by using HPLC analysis which gives information about the amount of individual carotenoid constituents in a sample.

Life-History Trade-off hypothesis

The life history trade-off hypothesis predicts a trade-off will be made between reproduction and survival when resources are scarce (Zera and Harshman 2001). I tested this hypothesis in terms of the carotenoid content in the red integument colouration representing reproduction versus the carotenoid content in the black, silver, and yellow integument and more notably, muscle tissue representing survival. Carotenoids have important somatic physiological functions such as immunity and antioxidant activity that could be particularly useful in muscle tissue (Britton et al. 2008). No trade-off was observed, although there is also evidence for this in the literature especially in studies that do not experimentally cause an immunological challenge (McGraw & Toomey 2010). However, to better test the trade-off hypothesis other tissue such as the liver, adipose tissue, gonads, seminal plasma, other organs, and circulating plasma should be tested for carotenoid content or an immune challenge should be introduced.

Source of carotenoids

Redside dace are known aerial feeders and their diet is composed primarily of terrestrial insects of the Order Diptera (Daniels & Wisniewski 1994, Schwartz & Norvel 1958). This study was novel in that it not only traced carotenoid allocation in a wild population of fish instead of a captive one, but it also determined the natural dietary source of carotenoids and the rarity of carotenoids in the system. Diptera made up the vast majority of insects near the redside feeding area at the surface of the water and Coleoptera were also present. Through carotenoid extraction of insects, it was found that Diptera and Coleoptera both contained carotenoids. This study was conducted over three days during the spawning season a year following the initial collection of redside dace.

This represents preliminary work to demonstrate that redside dace are capable of obtaining carotenoids through their insect diet and that each insect contains a low level of carotenoids. Therefore, the carotenoid-based colouration is likely indicative of foraging capability (Searcy & Nowicki 2005, Olson & Owens 1998, Smith & Harper 2003). In order to develop this idea, insects should be captured prior, during, and after the spawning season to determine seasonal variance of insect abundance and carotenoid content. Insects should also be captured at the same time as redside dace to be able to make more conclusive statements about diet. In addition, dietary manipulations using insects could be performed on captive redside dace to see how insect availability affects colouration using their true dietary source of carotenoids.

Future Directions

Breakdown of carotenoid content

As previously noted, breaking down the total carotenoid content reported here into the individual carotenoid constituents for the red integument could improve this study of reliable signalling as well as the allocation and trade-off study. The data currently takes into account the maximum absorbance of the sample which could be incorporating only the most abundant carotenoid, while other carotenoids could have lower absorbance values and therefore, their variation between individuals is not being considered at all. In order to break down the carotenoid constituents of the red integument as well as the other integument types and muscle, high performance liquid chromatography (HPLC) analysis will be performed following saponification to remove

esters (Toomey & McGraw 2007). In addition, this will be a good opportunity to compare carotenoid analysis performed via absorbance spectroscopy and HPLC analysis.

Experimentally manipulate signal

In order to gain further understanding of the effect of carotenoids on colouration in redside dace, dietary carotenoid manipulations could be performed. It would be particularly interesting to perform carotenoid supplementation of redside dace diet using Diptera since this is their most predominant natural source of carotenoids. In order to test the effect of carotenoids on colouration alone, the diet should be supplemented with carotenoids and reflectance spectrometry should be performed prior to and following supplementation. In zebra finches, spectral properties of the colouration on the beak increased as the diet was supplemented with carotenoids, although there was a colouration plateau at the highest levels of supplementation (Alonso-Alvarez et al. 2004). This study on zebra finches also incorporated an immunological challenge by injecting *E. coli* into the treatment group and determining how this affects colouration. It was found that the immune challenge diverted carotenoids from the plasma and colouration (see Alonso-Alvarez et al. 2004). Providing an immune challenge at different levels of carotenoid supplementation could help determine if there really is a life history trade-off occurring in redside dace.

Visual modeling

As discussed previously, when attempting to correlate carotenoid concentration with spectral properties of colouration, it is important to take into account the vision of

the species in question and to understand the constituent carotenoids that make up the colouration as well as the total carotenoid content of the colouration (Pike et al. 2011). In order to account for visual discrepancies, a visual model should be considered in which the peak sensitivity wavelengths of each of the cones of the eye of the redside dace are considered as was done previously in goldfish (Neumeyer 1985, Pike et al. 2011). In order to achieve this in goldfish, behaviour trials were conducted to determine the stimulation points of the cones by testing their response when given the choice of various wavelengths (Neumeyer 1985). Another method that was used in adult Nile tilapia (*Oreochromis niloticus*) was electrophysiological assessment. Tilapia were exposed to light in 20 nm intervals and their retinal response was measured using glass microelectrodes in order to find their peak sensitivities (Lisney et al. 2010). This process along with separating the constituent carotenoids will clarify the issue of the reliability of the spectral properties of the red integument as a signal of carotenoid content.

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