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EVIDENCE FOR CAROTENOID PIGMENTS IN THE FACIAL BANDS OF TWO MANGROVE CRAB SPECIES FROM SINGAPORE

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ABSTRACT. — The blue-green facial bands in two species of mangrove crabs, *Perisesarma eumolpe* (De Man) and *Perisesarma indiarum* (Tweedie) (Fig. 1), are known to be important in mate and/or species recognition and are believed to convey the physical 'quality' of the individual. For colour to be an effective indicator of quality, there has to be a direct production cost of the colour. Carotenoid-based pigments in animals fulfill these criteria. Being unable to biosynthesize carotenoids de novo, animals rely on dietary supply to achieve carotenoid-based pigmentation; therefore their presence can reflect foraging ability. Facial band tissues of *Perisesarma eumolpe* and *Perisesarma indiarum* were extracted and analysed for carotenoids using High Performance Liquid Chromatography (HPLC). The results confirm the presence of carotenoids in the facial bands of both species.

KEY WORDS. — facial band, crabs, *Perisesarma*, carotenoid, HPLC

INTRODUCTION

Colouration plays a critical role in a number animal interactions, e.g., as warning signals, camouflage, or indicators of social status and/or mate quality (Endler, 1978; Todd et al., 2005; Vasquez & Pfennig, 2007; Pryke & Andersson, 2003; Mougeot et al., 2009). The physiological processes through which certain species develop colour can hold important clues related to the function of that particular visual cue. In order for colour to be an effective indicator of mate quality, for example, there has to be a direct production cost of the colour (Pryke & Andersson, 2003; Mougeot et al., 2009). Individuals that can afford to channel resources (that could otherwise be used for physiological and immunological functions) towards the production of seemingly extravagant, colourful, sexual signals will be judged to be more competent and thus attractive (Zahavi, 1975, 1991). Carotenoid-based pigments in animals fulfill these criteria, as their high production costs enforce the reliability of colour signals (Moller et al., 2000). Carotenoids are lipid-soluble pigments, synthesized from geranyl-geranyl diphosphate by all photosynthetic organisms (Baron et al., 2008), including algae. Animals do not possess the ability to biosynthesize carotenoids de novo (McGraw et al., 2003; Chatzifotis et al., 2005), hence they can only rely on dietary supply to achieve carotenoid-based pigmentation. The intensity of such colours may therefore represent the foraging efficiency of the signaller (Moller et al., 2000).

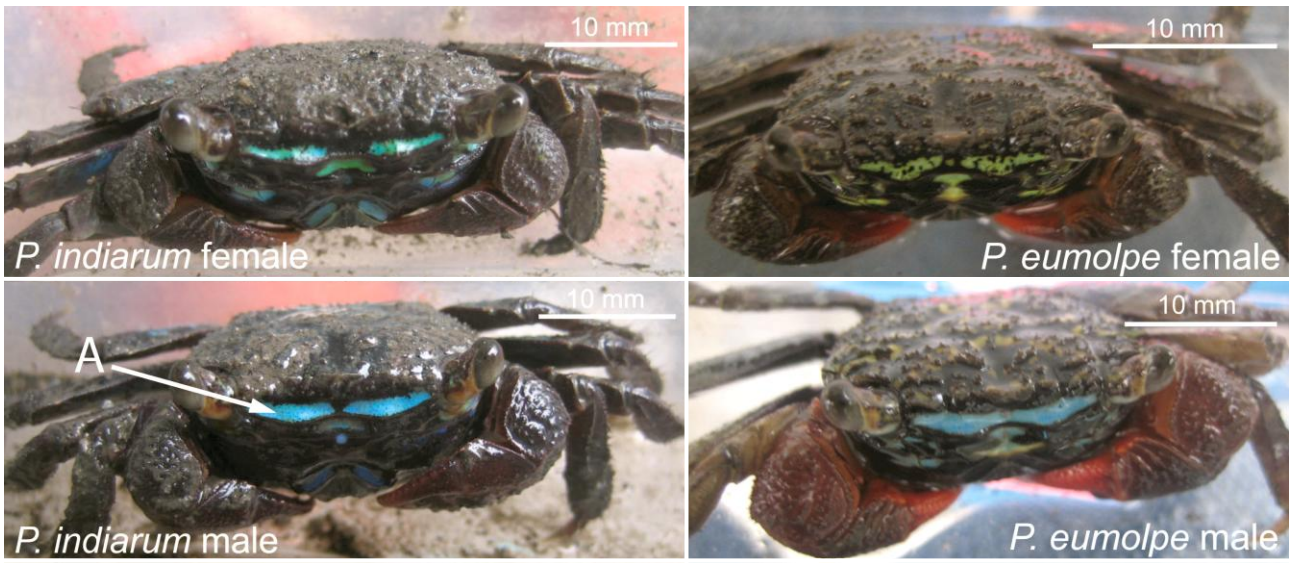

Fig. 1. The blue-green facial bands (A) of the two species of *Perisesarma* studied.

Mangrove crabs of the genus *Perisesarma* are common throughout the Indo-West Pacific. Many exhibit conspicuously coloured facial bands, and this phenomenon has been studied in detail for two species found in Singapore's mangroves, *Perisesarma eumolpe* (De Man) and *Perisesarma indiarum* (Tweedie). Huang et al. (2007) determined that the blue-green bands varied both between and within these two species. Todd et al. (2011) showed how the bands are important in mate and/or species recognition and can also indicate the physical 'quality' of the individual. Todd et al. (2011) proposed that the facial band pigments are diet-derived and carotenoid-based because, when test crabs were starved, their brightness and saturation decreased significantly. The chemical composition of these pigments, however, has not yet been investigated directly. Therefore, in the present study, we extracted facial band pigments from both *Perisesarma eumolpe* and *Perisesarma indiarum* and used established laboratory techniques to test the hypothesis that carotenoids are present.

MATERIAL AND METHODS

Specimen collection. — *Perisesarma eumolpe* (De Man) and *Perisesarma indiarum* (Tweedie) were collected from Sungei Mandai Kechil mangrove forest (1°26′N, 103°45′E), Singapore. These were maintained in separate clear plastic tanks (29 × 18 × 18 cm) containing mangrove mud (at least 3 cm in depth). Tanks were immersed in seawater (at least 1 cm in depth), which kept the mud moist by penetrating through the perforated tank bases (following Boon et al., 2008). Crab tanks were housed in an indoor holding area with natural ventilation and controlled light settings that resembled a normal local (12:12 light:dark) photo-period.

Pigment characterisation. — To test for the presence of carotenoids in the facial bands of both species and sexes, acidified pyridine and solvent transfer tests were conducted according to the procedures stipulated by McGraw et al. (2005). 3–5 mg of the major colour face bands, including underlying tissue, were collected from crabs (five crabs per sex and species, 20 crabs in total) that had been frozen. Owing to a limited sample size for each species, band samples from both sexes were pooled for the pigment extraction but separated according to species. Fractions of the carapace tissue without colour bands, i.e., around the rostrum (a dark grey-blue area), were prepared in the same manner for comparison. A sample containing 5 mg of an *Avicennia alba* leaf, one of the crabs' main food items (Boon et al., 2008), was also included in subsequent chemical tests of peak profiles so that comparisons with the carapace HPLC profiles could be made. Identical peaks between the *Avicennia* profile and face band tissue profiles would indicate the presence of similar pigment compounds.

One ml of pyridine, acidified with concentrated hydrochloric acid (HCl), was added to all sample tubes. The tubes of tissue and pyridine were then capped tightly and placed in a 95°C water bath for 4 h. This extraction technique works by weakening non-covalent hydrogen bonds that bind the pigments to proteins, especially in carotenoproteins, thereby releasing them into the pyridine solution. Tubes were allowed to cool to room temperature before handling. If carotenoids were present in the tissue, the heated colourless pyridine solution would be colourful after the treatment.

A supplementary chemical procedure was conducted to further test the presence of carotenoids in the facial band tissue. Lipid-soluble compounds, including carotenoid pigments, transfer readily to strong, non-polar organic solvents such as hexane (for non-polar carotenoids) and tert-butyl methyl ether (TBME) (for polar carotenoids) (McGraw, 2005). Colourants of different chemical composition, such as melanins and porphyrins, tend to be soluble in aqueous solutions, under either acidic or basic conditions. Partitioning water and lipid-soluble components between aqueous pyridine and hexane:TBME solvents works on this fundamental concept. 2 ml of distilled water was first added to each of the tubes from the previous procedure. The capped tube was inverted repeatedly to homogenize the mixture. 1 ml of hexane:TBME solution (1:1 proportion) was then added to every tube. Each tube was capped and shaken vigorously for 2 min to ensure the two phases mixed well. To fully separate the hexane:TBME from the now-aqueous pyridine, the tubes were left to stand overnight at room temperature with no light (by wrapping the tubes in aluminium foil).

The carotenoids extracted from the procedure described above were further analysed using High Performance Liquid Chromatography (HPLC). The supernatant from each mixture was first transferred to a clean tube, after which the solvent was evaporated to dryness under a stream of nitrogen. The pigment residue was re-suspended in 200 µl of HPLC mobile phase (methanol:acetonitrile:chloroform, 46:46:8) prior to analysis, in accordance to a protocol stipulated by McGraw et al. (2001, 2003). 50 µl of each re-suspended sample was injected into a Waters™ 600E Autosampler Water Pump HPLC (Millipore Corp.) fitted with a Develosil RPAqueous RP-30 column, and an Eppendorf TC-50 column heater set at 27°C. An isocratic (solvent composition constant during separation) system at a constant flow rate of 0.3 ml min^{-1} was utilised and run for 60 min for every sample, allowing sufficient time for carotenoids to elute if present. Data was collected over wavelengths 300–700 nm using a Waters™ 996 Photodiode Array (PDA) detector and processed using Empower™ software. Pigment compounds, represented by absorbance peaks in the HPLC chromatogram, could be identified by comparing their respective retention times and absorbance maxima to those of authentic reference carotenoids, i.e., lutein, retinoic acid, astaxanthin, β–carotene, and mixed isomers of carotene obtained from carrots, run as external standards.

Mixtures of various pigments with highly similar retention times and absorbance peaks were further resolved using an additional HPLC–Ultra-violet/visible light (HPLC–UV/VIS) system (Waters™ 1525µ Water Pump Binary System HPLC connected to a UV/VIS Detector 2487). Pigment profiles derived from the previous analysis using the PDA detector were used to estimate two specific wavelengths at which most HPLC peaks could be detected. Each pigment sample was then run under the same parameters as the previous procedure, but using the binary wavelength detector instead of the PDA. This detector system is more sensitive than the PDA at resolving individual HPLC peaks corresponding to the elution of different pigment compounds, collecting data at two user-specified wavelengths. Pigment profiles derived from all samples were assessed qualitatively.

RESULTS

Carotenoids were present in all samples tested, including fractions without colour band tissue. This was evidenced by the coloured organic solution obtained from each fraction in the pyridine-hexane:TBME extraction protocol preceding isocratic HPLC analysis. Carotenoid elution profiles were not well-resolved using the photodiode array (PDA) detector because of the high concentration of different carotenoids within each sample that tended to elute at identical times. Most carotenoids in each sample absorbed within the range of 360–468 nm, and resolution of elution peaks appeared highest at the wavelengths of 360 nm and 468 nm. Thus these two wavelengths were chosen for subsequent isocratic runs using the UV/visible detector.

Qualitatively, chromatograms plotted at 468 nm indicated similar carotenoid contents for extracts from each species, with two major peaks representing dominant carotenoids eluting at identical times (Figs. 2, 3). Negative (without facial band tissue) fractions, however, yielded elution profiles differing in one common major peak (mean elution time: 2.618 ± 0.044) that is prominent in the positive (containing facial band tissue) fractions. This peak is either too weak to be resolved or entirely absent from the *Perisesarma eumolpe* profile (Fig. 2a). In the *Perisesarma indiarum* profile, however, the peak (1) was still present, but its relative intensity (absorbance) as compared to the other dominant peak (2) was much reduced (Fig. 3a). Comparison of sample chromatograms against carotenoid profiles of pure standards did not allow for unambiguous identification of the compound represented by the missing peak, since the mixtures of carotenoids were not well separated by HPLC. The same problem of poor separation also prevented the exact matching of peaks from the *Avicennia alba* leaf extract (Fig. 4) to those from the crab extracts. Hence it cannot be ascertained whether the carotenoids in the facial bands are directly derived from the crabs' diet of *Avicennia alba* leaves.

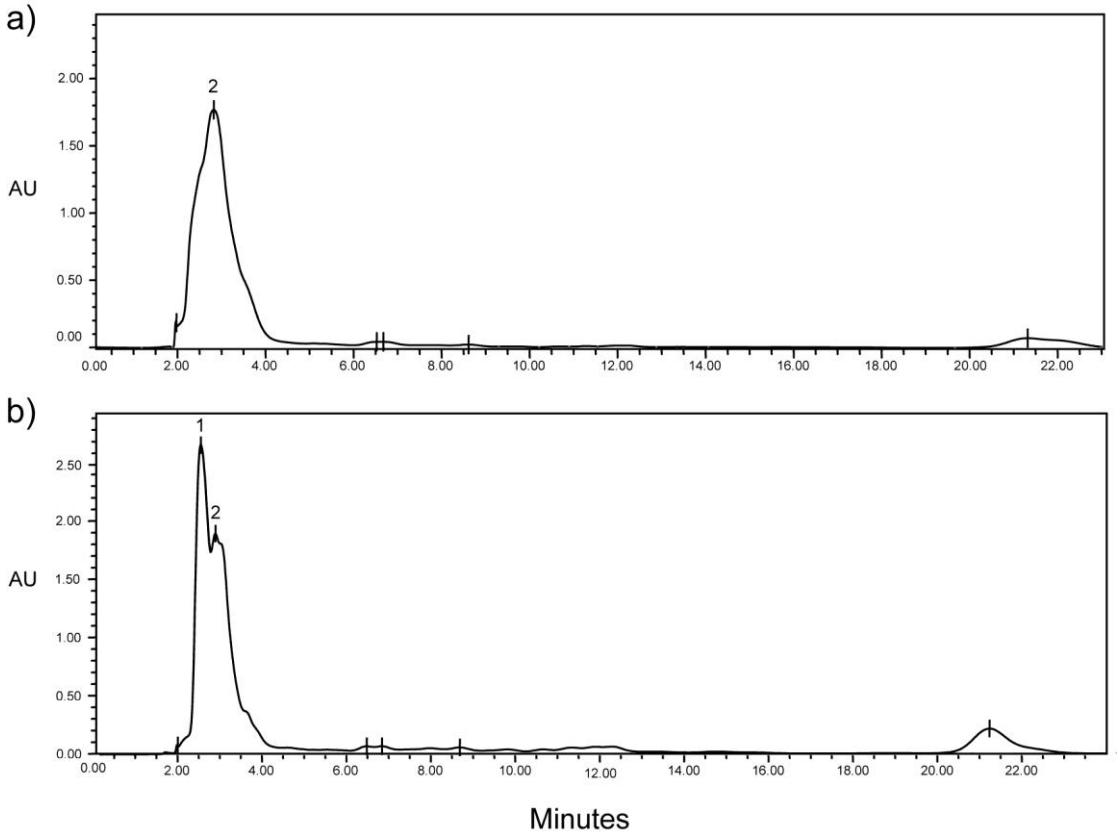

Fig. 2. Chromatogram plotted at 468 nm showing HPLC carotenoid elution profile of (a) *Perisesarma eumolpe* mixed fraction without facial band and (b) *Perisesarma eumolpe* mixed fraction with facial band. Numbers 1 and 2 denote dominant peaks on each profile. Other identical features are marked by vertical strokes.

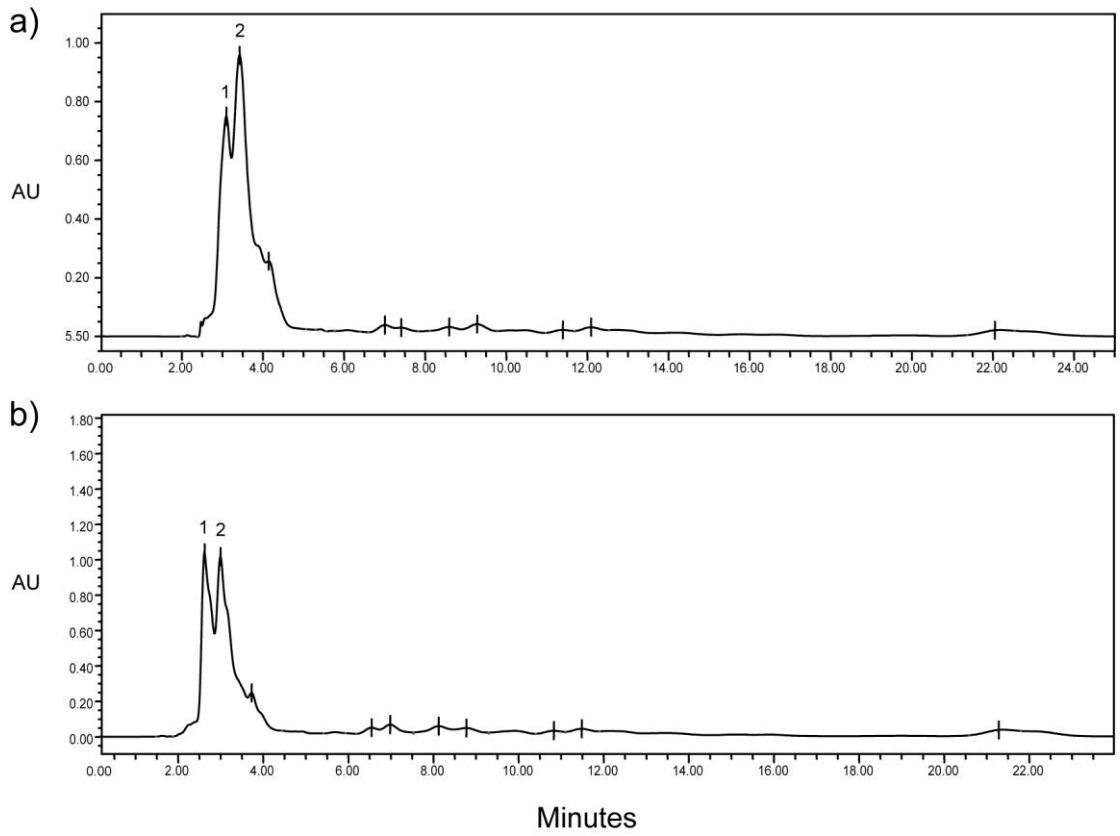

Fig. 3. Chromatogram plotted at 468 nm showing HPLC carotenoid elution profile of (a) *Perisesarma indiarum* mixed fraction without facial band and (b) *Perisesarma indiarum* mixed fraction with facial band. Numbers 1 and 2 denote dominant peaks on each profile. Other identical features are marked by vertical strokes.

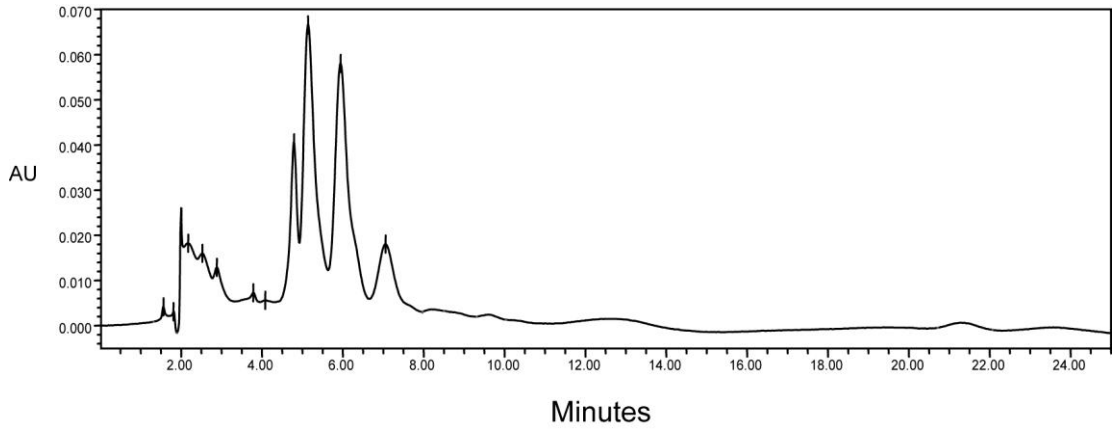

Fig. 4. Chromatogram plotted at 468 nm showing HPLC carotenoid elution profile of *Avicennia alba* leaf extract.

DISCUSSION

The starvation-induced changes in facial band saturation and brightness determined by Todd et al. (2011) suggested that the pigments involved are primarily carotenoid in nature. The High Performance Liquid Chromatography (HPLC) performed here reveals the presence of a particular carotenoid in the crab carapace (including underlying epithelial tissue) fractions with facial bands intact. It is represented on each positive elution profile as a single, relatively dominant, peak with high absorbance and consistent retention times across samples. This peak, however, does not match with any one of the known external standards, which means that the compound is not astaxanthin, lutein, retinoic acid, or β–carotene. Nevertheless, it is possible that the single peak actually encompasses multiple peaks if viewed at a higher resolution than we were able to achieve. If this was the case, it would indicate that more than one carotenoid is responsible for the colouration in the crabs' facial bands. Better separation of compounds and more refined peaks might be obtained using gradient flow elution methods (instead of isocratic flow), in which the solvent or eluent composition is gradually changed during the analysis (Gaillard et al., 2004).

The carotenoid compounds exclusive to the facial band that were extracted using the thermo–chemical solvent transfer method of McGraw (2005), and which produce prominent peaks in HPLC elution profiles, most probably do not represent the original form of face band carotenoids in vivo. Carotenoids are known to be both light- and oxygen-sensitive; exposure to either factor during extraction could have resulted in their rapid oxidation or degradation (de Ritter & Purcell, 1981). Also, the carotenoid extraction procedures used in this study involve heating the tissue fractions at a high temperature (95°C), which will weaken and break the non-covalent bonds between carotenoids and proteins in carotenoproteins (McGraw et al., 2005). Hence, the extracted facial band carotenoids may not represent the actual pigment compound in situ, since they could merely be prosthetic groups removed from a coloured, proteinaceous compound that has lost its initial light-reflective or absorptive properties upon protein degradation (Zagalsky, 1985). Nevertheless, we can infer from the HPLC results presented here, and the food deprivation experiments of Todd et al. (2011), that synthesis of the facial band pigment is probably dependent on the availability of carotenoids, which the crabs can only obtain from consumption of mangrove plant leaves.

In various species, manipulations of carotenoid diets have been shown to significantly influence pigmentation. For example, nestling white storks (*Ciconia ciconia*) that consume red swamp crayfish (*Procambarus clarkii*), a rich source of crustacean astaxanthin, develop intensely orange astaxanthin-based skin pigmentation, whilst those that do not consume the crayfish have white or un-pigmented skin (Negro et al., 2000). Male guppies (*Poecilia reticulata*) that are fed carotenoid-enhanced diets also develop prominent red and orange spots that are a few-fold brighter and more saturated in colour compared to males who are fed a basal diet without added carotenoids (Grether et al., 2001). The evident relationship between carotenoid pigment concentration and integumentary colour intensity from these examples show how dietary manipulation in *Perisesarma eumolpe* and *Perisesarma indiarum* could cause the changes in facial colour band intensity and saturation observed by Todd et al. (2011).

CONCLUSIONS

HPLC analysis of pigment extracts indicates the presence of carotenoids that are exclusive to the facial bands of *Perisesarma eumolpe* and *Perisesarma indiarum*, but further, higher resolution, work is required to determine exactly which compounds are present. Since animal carotenoids can only be obtained from dietary sources, production is most likely to be associated with diet—a supposition supported by Todd et al. (2011). The relationship between diet and pigment could potentially be tested by measuring the quantity of carotenoid present under different crab-feeding regimes. Carotenoid-based colouration is influenced by parasitism and the overall condition, as well as nutrition in a wide range of species (e.g., Borgia & Collis, 1989; Hill & McGraw, 2004; Clotfelter et al., 2007; Thorogood et al., 2008), and the intensity of such colours can accurately reflect the foraging efficiency and health of the signaller (Moller et al., 2000). The evidence to date (e.g., Huang et al., 2007; Todd et al., 2011; and the present study) strongly suggests that facial band pigments are carotenoid-based and contribute towards intra- and inter-specific signalling in *Perisesarma eumolpe* and *Perisesarma indiarum*.

LITERATURE CITED

Baron, M., S. Davies, L. Alexander, D. Snellgrove & K. A. Sloman, 2008. The effect of dietary pigments on the coloration and behaviour of flame-red dwarf gourami, *Colisa lalia*. *Animal Behaviour*, **75**: 1041–1051.

Boon, P. Y., D. C. J. Yeo & P. A. Todd, 2008. Feeding ecology of the genus *Perisesarma* (Crustacea: Decapoda: Brachyura: Sesarmidae) in Mandai Mangroves, Singapore. *Journal of Crustacean Biology*, **28**: 480–484.

Borgia, G. & K. Collis, 1989. Female choice for parasite-free male satin bowerbirds and the evolution of bright male plumage. *Behavioral Ecology and Sociobiology*, **25**: 445–453.

Chatzifotis, S., M. Pavlidis, C. Donate Jimenso, G. Vardanis, A. Sterioti & P. Divanach, 2005. The effect of different carotenoids on skin coloration of cultured red porgy (*Pagrus pagrus*). *Aquaculture Research*, **36**: 1517–1525.

Clotfelter, E. D., D. R. Ardia & K. J. McGraw, 2007. Red fish, blue fish: Trade-offs between pigmentation and immunity in *Betta splendens*. *Behavioral Ecology*, **18**: 1139–1145.

de Ritter, E. & A. E. Purcell, 1981. Carotenoid analytical methods. In: Bauernfeind, J. C. (ed.), *Carotenoids as Colorants and Vitamin A Precursors*. Academic Press Inc, New York. Pp. 815–917.

Endler, J. A., 1978. A predator's view of animal color patterns. *Evolutionary Biology*, **11**: 319–364.

Gaillard, M., J. Cedrik, F. Cezilly & M. Perrot-Minnot, 2004. Carotenoids of two freshwater amphipod species (*Gammarus pulex* and *G. roeseli*) and their common acanthocephalan parasite *Polymorphus minutus*. *Comparative Biochemistry and Physiology Part B*: *Biochemistry and Molecular Biology*, **139**: 129–136.

Grether, G. F., J. Hudon & J. A. Endler, 2001. Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*). *Proceedings of the Royal Society Biological Sciences*, **268**(1473): 1245–1253.

Hill, G. E. & K. J. McGraw, 2004. Correlated changes in male plumage coloration and female mate choice in cardueline finches. *Animal Behaviour*, **67**: 27–35.

Huang, H., P. A. Todd & D. C. J. Yeo, 2007. Inter- and intra-specific variation in the facial colours of *Perisesarma eumolpe* and *Perisesarma indiarum* (Crustacea: Brachyura: Sesarmidae). *Hydrobiologia*, **598**: 361–371.

McGraw, K. J., G. E. Hill, R. Stradi & R. S. Parker, 2001. The influence of carotenoid acquisition and utilization on the maintenance of species-typical plumage pigmentation in male American goldfinches (*Carduelis tristis*) and northern cardinals (*Cardinalis cardinalis*). *Physiological and Biochemical Zoology*, **74**: 843–852.

McGraw, K. J., G. E. Hill & R. S. Parker, 2003. Carotenoid pigments in a mutant cardinal: Implications for the genetic and enzymatic control mechanisms of carotenoid metabolism in birds. *The Condor*, **105**: 587–592.

McGraw, K. J., 2005. Interspecific variation in dietary carotenoid assimilation in birds: Links to phylogeny and color ornamentation. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **142**: 245–250.

McGraw, K. J. & G. E. Hill, 2001. Carotenoid access and intraspecific variation in plumage pigmentation in American goldfinches (*Carduelis tristis*) and Northern cardinals (*Cardinalis cardinalis*). *Functional Ecology*, **15**: 732–739.

McGraw, K. J., J. Hudon, G. E. Hill & R. S. Parker, 2005. A simple and inexpensive chemical test for behavioral ecologists to determine the presence of carotenoid pigments in animal tissues. *Behavioral Ecology and Sociobiology*, **57**: 391–397.

Møller, A. P., C. Biard, J. D. Blount, D. C. Houston, P. Ninni, N. Saino & P. F. Surai, 2000. Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? *Poultry and Avian Biology Reviews*, **11**: 137–159.

Mougeot, F., L. Perez-Rodriguez, N. Sumozas & J. Terraube, 2009. Parasites, condition, immune responsiveness and carotenoid-based ornamentation in male red-legged partridge *Alectoris rufa*. *Journal of Avian Biology*, **40**: 67–74.

Negro, J. J., J. L. Tella, G. Blanco, M. G. Forero & J. Garrido-Fernandez, 2000. Diet explains interpopulation variation of plasma carotenoids and skin pigmentation in nestling white storks. *Physiological and Biochemical Zoology*, **73**: 97–101.

Pryke, S. R. & S. Andersson, 2003. Carotenoid-based epaulettes reveal male competitive ability: Experiments with resident and floater red-shouldered widowbirds. *Animal Behaviour*, **66**: 217–224.

Thorogood, R., R. M. Kilner, F. Karadas & J. G. Ewen, 2008. Spectral mouth colour of nestlings changes with carotenoid availability. *Functional Ecology*, **22**: 1044–1051.

Todd, P. A., R. J. Ladle, R. Briers & A. Brunton, 2005. Quantifying two-dimensional dichromatic patterns using a photographic technique: case study on the shore crab (*Carcinus maenas* L.). *Ecological Research*, **20**: 497–501.

Todd, P. A., W. Y. Wang, H. Huang, C. C. Belle, M. L. M. Lim & D. C. J. Yeo, 2011. The function of colourful facial bands in mangrove crab (*Perisesarma*) communication. *Journal of Experimental Marine Biology and Ecology*, **407**: 26–33.

Vasquez, T. & K. S. Pfennig, 2007. Looking on the bright side: Females prefer coloration indicative of male size and condition in the sexually dichromatic spadefoot toad, *Scaphiopus couchii*. *Behavioral Ecology and Sociobiology*, **62**: 127–135.

Zagalsky, P. F., 1985. Invertebrate carotenoproteins. *Methods in Enzymology*, **111**: 216–247.

Zahavi, A., 1975. Mate selection-a selection for a handicap. *Journal of Theoretical Biology*, **53**: 205–214.

Zahavi, A., 1991. On the definition of sexual selection, Fisher's model, and the evolution of waste and of signals in general. *Animal Behaviour*, **42**: 501–503.