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Cite this article: Twyman H, Valenzuela N, Literman R, Andersson S, Mundy NI. 2016 Seeing red to being red: conserved genetic mechanism for red cone oil droplets and co-option for red coloration in birds and turtles. *Proc. R. Soc. B* **283**: 20161208. http://dx.doi.org/10.1098/rspb.2016.1208

Received: 1 June 2016
Accepted: 14 July 2016

Subject Areas:
evolution, genetics

Keywords:
carotenoid coloration, turtles, birds, *CYP2J*, colour vision, retinal oil droplets

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2016.1208 or via http://rspb.royalsocietypublishing.org.

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Seeing red to being red: conserved genetic mechanism for red cone oil droplets and co-option for red coloration in birds and turtles

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Avian ketocarotenoid pigments occur in both the red retinal oil droplets that contribute to colour vision and bright red coloration used in signalling. Turtles are the only other tetrapods with red retinal oil droplets, and some also display red carotenoid-based coloration. Recently, the *CYP2J19* gene was strongly implicated in ketocarotenoid synthesis in birds. Here, we investigate *CYP2J19* evolution in relation to colour vision and red coloration in reptiles using genomic and expression data. We show that turtles, but not crocodiles or lepidosaurs, possess a *CYP2J19* orthologue, which arose via gene duplication before turtles and archosaurs split, and which is strongly and specifically expressed in the ketocarotenoid-containing retina and red integument. We infer that *CYP2J19* initially functioned in colour vision in archelosaurs and conclude that red ketocarotenoid-based coloration evolved independently in birds and turtles via gene regulatory changes of *CYP2J19*. Our results suggest that red oil droplets contributed to colour vision in dinosaurs and pterosaurs.

1. Introduction

Ketocarotenoids are red xanthophyll carotenoids whose terminal ketone groups, when on the C4 position on one or both end rings, lead to the absorption of light at longer wavelengths than in other xanthophylls [1]. They have been extensively studied in birds where they are the predominant mechanism of saturated red colours that are frequently sexually or socially selected signals [2–5]. In addition, ketocarotenoids perform another key function in birds: the ketocarotenoid astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione) is the red pigment found in the oil droplets in long-wave sensitive (LWS) cones of the avian retina [6], where they act as cut-off filters to enhance colour discrimination [7–10]. Ketocarotenoids in most land birds are not obtained directly from the diet but are instead synthesized by ketolation (addition of double-bonded oxygen at the C4 position of the end ring) of dietary yellow carotenoids such as zeaxanthin [1]. The mechanism was for a long time obscure, but recent studies have strongly implicated a locus encoding a cytochrome P450 monooxygenase (*CYP2J19*) as the main or only avian ketolase enzyme that catalyses the conversion of dietary xanthophylls to the ketocarotenoids that are deployed in both red integumentary coloration and red retinal oil droplets [11,12]. This raises the intriguing question of the origin of *CYP2J19* function in birds, and its ancestry in vertebrates more generally.

Turtles (Testudines) are the only group of tetrapods apart from birds that possess red retinal oil droplets [6,13,14], and these probably contain ketocarotenoids [15,16]. By contrast, other extant reptile lineages either lack retinal oil

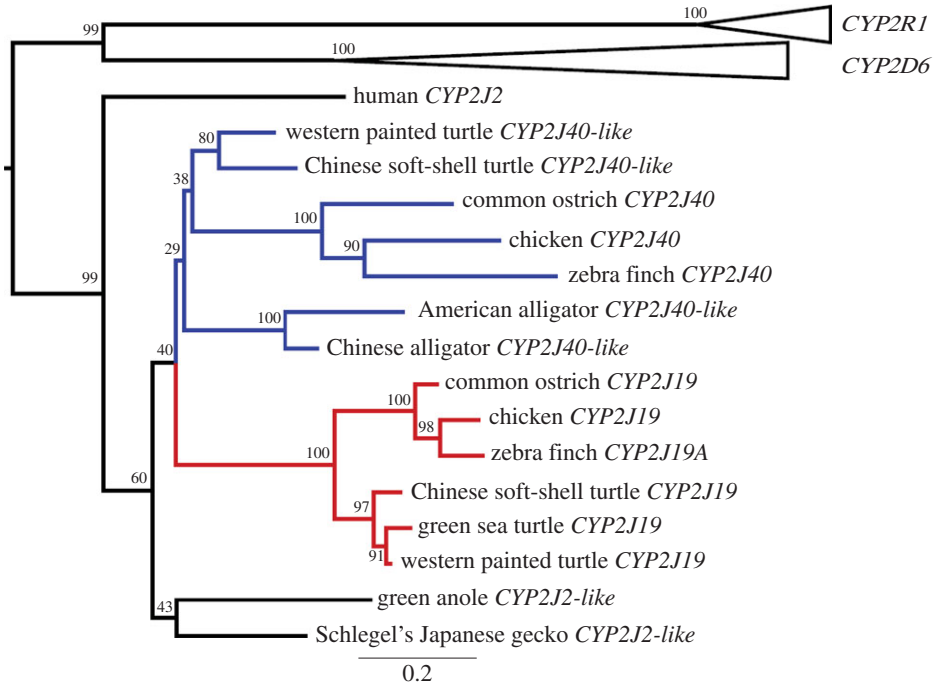

Figure 1. JTT model-based protein phylogeny based on all *CYP2J*, *CYP2R1* and *CYP2D6* sequences obtained. Bootstrap values are based on 1000 pseudoreplicates. *CYP2J19*-like sequences and *CYP2J40*-like sequences are outlined in red and blue, respectively. *CYP2R1* and *CYP2D6* clades have been collapsed. For fully expanded phylogeny, see the electronic supplementary material, figure S1.

droplets entirely (crocodiles, snakes) or have coloured oil droplets varying from clear to yellow and green, but not red (many lizards) [14]. Like birds, turtles have excellent tetrachromatic colour vision [17,18].

Red coloration of the integument is uncommon in turtles, but red coloration specifically due to the presence of ketocarotenoids has been reported [19] and shown to be related to sexual selection [20]. Importantly, aquatic turtles, unlike most land birds, have the potential to obtain red ketocarotenoids directly from their diet [21,22], and whether turtles have the capacity for carotenoid ketolation remains unknown.

Several interesting questions arise: is there a shared genetic basis for ketocarotenoid synthesis in birds and turtles? Alternatively, was ketocarotenoid synthesis independently derived in the two groups, or even absent in turtles? If ketocarotenoid synthesis does have a shared genetic basis, was the original function of *CYP2J19* for colour vision or coloration?

The phylogenetic position of turtles within the reptiles has long been a source of debate [23] mainly due to the highly derived morphologies of turtles and the somewhat contradictory molecular evidence [24,25]. More recently, a number of large-scale molecular studies using genomic data support a sister relationship between turtles and the archosaurs, forming the turtle–archosaur clade (Archelosauria) independent from the lepidosaurs (lizards, snakes and tuatara) [26–31]. With turtles now placed as an outgroup to archosaurs, the evolution of a locus related to colour vision and coloration in turtles has the potential to inform the occurrence of these traits in the dinosaurs and pterosaurs.

Here, we address the genetic basis of ketocarotenoids in the retina and integument of turtles and birds using two approaches. First, we mine recent genomic data and perform phylogenetic analyses to reconstruct the evolutionary history of *CYP2J19* within reptiles and birds. Second, we examine expression of *CYP2J*-like loci in the western painted turtle (*Chrysemys picta bellii*), which possesses both red retinal oil droplets and red ketocarotenoid-based integumentary coloration, to obtain evidence for functional involvement of *CYP2J19* in ketocarotenoid synthesis in different tissues in Testudines. We find that *CYP2J19* is conserved in birds and turtles but has apparently been lost in crocodiles, and that patterns of *CYP2J19* expression are consistent with a function in both colour vision and coloration in turtles, as in birds. We interpret these results to suggest that the carotenoid ketolase *CYP2J19* originally functioned in colour vision in the Testudines–archosaur clade ancestor, and has since been independently and repeatedly recruited for red integumental coloration by changes in gene regulation in turtles and birds. Finally, our results imply the presence of red retinal oil droplets in lineages related to the birds, including dinosaurs and pterosaurs, which provides new insights into the reconstruction of the evolution of colour vision in these groups.

2. Results

(a) Evolution of *CYP2J* genes in amniotes

BLASTn searches revealed the presence of two *CYP2J* family loci in the three most complete turtle genomes out of the four available (western painted turtle, Chinese soft-shell turtle and green sea turtle) [28,29]. By contrast, we found at most a single *CYP2J* sequence in crocodilians (American alligator, Chinese alligator) and lepidosaurs (green anole, Schlegel's Japanese gecko). Strikingly, phylogenetic analyses revealed that one of the *CYP2J* loci in turtles is orthologous to avian *CYP2J19*, because turtle sequences form a well-supported (100% bootstrap support) monophyletic clade with *CYP2J19* sequences from birds (figure 1). No *CYP2J19* orthologues were identified in either crocodilians or lepidosaurs. The remaining reptile and avian *CYP2J* loci group together by order but support for other relationships is variable. Reconstructions suggest that the crocodilian and second turtle

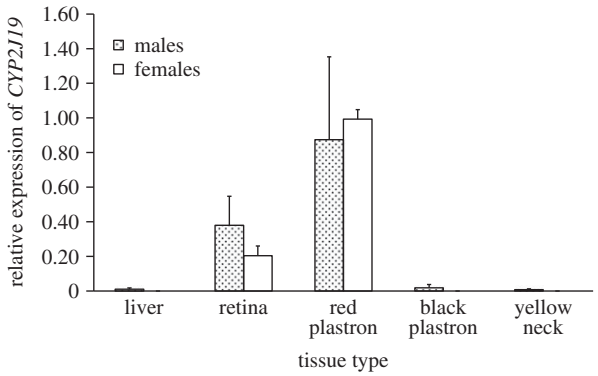

Figure 2. Results of qRT-PCR experiments quantifying male and female tissue-specific expression for *CYP2J19* normalized against *TBP*, *GAPDH* and *HPRT1*. Error bars represent s.e.m. from three individual males and females.

CYP2J locus are related to avian *CYP2J40*, but the bootstrap support is weak. The single *CYP2J* locus in lepidosaurs is placed as an outgroup to the Testudines and archosaur (crocodile + bird) sequences, with moderate bootstrap support (60%). The single human *CYP2J* locus (*CYP2J2*) is clearly an outgroup to all of the reptile and avian sequences (99% bootstrap support).

Overall therefore, we find strong evidence for the conservation of the *CYP2J19* locus in avian and turtle (Testudines) lineages, but no evidence for the presence of *CYP2J19* in crocodilians or lepidosaurs. Furthermore, our phylogenetic analysis indicates that *CYP2J19* arose from a duplication event that occurred after the divergence of reptiles and mammals and before the split between Testudines and archosaurs and *CYP2J19* was subsequently lost in crocodilians.

(b) Specific expression of *CYP2J19* in tissues containing red ketocarotenoids in the western painted turtle

Preliminary experiments revealed high *CYP2J19* expression in the retina and red plastron of western painted turtles of both sexes, and lower, variable expression in the liver, black plastron, yellow tail, black tail, yellow neck and black neck regions (not shown). By contrast, *CYP2J40* expression was detected in all tissue types (not shown).

Quantification of expression via qRT-PCR confirmed the presence of high *CYP2J19* expression in the ketocarotenoid-containing tissues (retina, red plastron) of both sexes, and low expression in other tissues (figure 2). There was significant heterogeneity of *CYP2J19* expression level among different tissue types ($F_{4,25} = 35.83$, $p < 0.001$) but not among sexes ($F_{1,28} = 0.4524$, $p = 0.51$) (table 1). As no sex-biased expression was detected, a comparison between all tissue types pooled for both sexes was carried out. The red plastron had the highest *CYP2J19* expression relative to all other tissues ($p < 0.05$), while the retina had higher expression when compared with all tissues except for the red plastron ($p < 0.001$) (table 1).

Table 1. Tukey's pairwise tests of *CYP2J19* expression.

pairwise tissue comparisons	*p* adj
liver, retina	0.0000420***
yellow neck, retina	0.0000284***
black plastron, retina	0.0000188***
red plastron, retina	0.0434566*
yellow neck, liver	0.9998532
black plastron, liver	0.9975022
red plastron, liver	0.0000000***
black plastron, yellow neck	0.9998199
red plastron, yellow neck	0.0000000***
red plastron, black plastron	0.0000000***

3. Discussion

We provide compelling evidence for the presence of a *CYP2J19* orthologue in three divergent Testudines lineages that suggests conservation of *CYP2J19* function in turtles and birds, despite approximately 250 million year divergence between these lineages [27]. Expression patterns of *CYP2J19* in painted turtles are consistent with this gene playing a role in ketocarotenoid synthesis in both retina and red-coloured integument, as recently described in birds [11,12]. This raises the question of whether the original function of *CYP2J19* was for colour vision or coloration. Consideration of the likely ancestral traits, given the extant distributions, strongly favours colour vision as the original function, as we now discuss (figure 3).

Colour vision involving ketocarotenoid-containing oil droplets in LWS cones appears to be a pervasive feature in almost all birds and turtles [6,15,16], implying that red oil droplets were present in both ancestral birds and ancestral turtles. By contrast, red ketocarotenoid coloration has a patchy distribution in both groups. In turtles, red coloration is sparsely distributed among both the pleurodirans and cryptodirans. Redness appears to be rare or absent in most turtle families except for two cryptodiran families, Emydidae and Geomydidae, where red coloration is more common, as well as several taxa within the pleurodiran family Chelidae. In addition, the pigmentary basis for redness in turtles has not been widely characterized. In birds, reconstruction of carotenoid-based plumage coloration has shown that this trait is not ancestral but was acquired multiple times during avian evolution [32]. Evolutionary reconstructions of carotenoid coloration in bare body parts (such as bills) in birds are still awaited. An independent line of evidence suggesting that carotenoid coloration in the integument was not ancestral in birds concerns their lack of specific pigment cells containing carotenoids. Reptiles, like amphibians and teleosts, express carotenoids (along with pteridines) in specialized xanthophores/erythrophores [33], whereas birds express integumentary carotenoids in keratinocytes. Although studies have found pigment cells in the avian iris that resemble reptilian xanthophores and iridophores [34,35], the absence of specialized pigment cells for carotenoids in the integument of birds seems to imply a period in their ancestry when carotenoids were not used for coloration.

As tetrachromatic colour vision involving red retinal oil droplets was probably present in the ancestral turtle and avian lineages, while red carotenoid-based coloration was probably absent in both, we conclude that the ancestral function of *CYP2J19* was for production of ketocarotenoids in red retinal oil droplets and that the role of *CYP2J19* in red carotenoid-based coloration evolved independently in the two lineages (figure 3). Many examples of phenotypic convergence via similar genetic changes have been observed across the

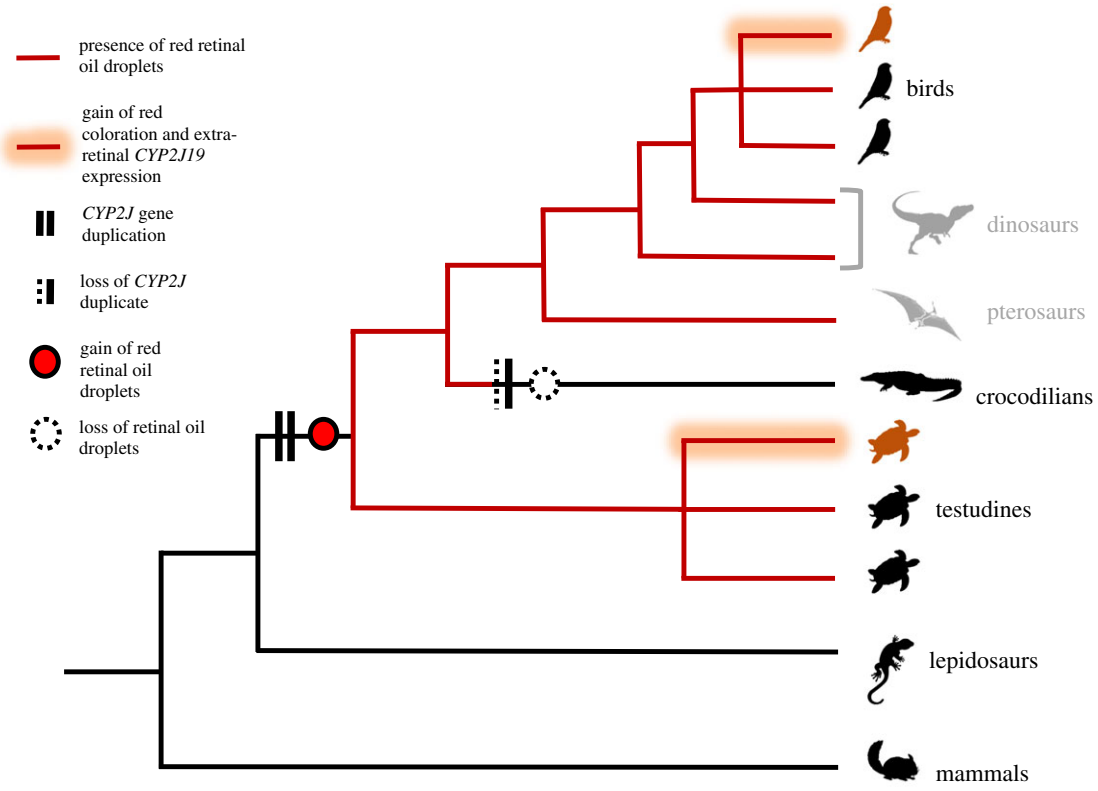

Figure 3. Reconstructed scenario for evolution of *CYP2J19* function in red retinal oil droplets and red ketocarotenoid integumentary coloration in reptiles. Red lines denote the presence of red retinal oil droplets. The inferred presence of ketocarotenoid-containing retinal oil droplets coincides with the inferred duplication of *CYP2J2* prior to the turtle–archosaur split, followed by subsequent loss of oil droplets and *CYP2J19* in the crocodilian lineage. Red highlighted branches show the independent gain of red ketocarotenoid-based coloration in certain turtle and bird lineages associated with co-option of *CYP2J19* expression for coloration.

vertebrates [36–38]. Several examples involve melanin pigmentation, and include the *MC1R* locus, which has been involved in convergent evolution of pale/dark coloration in birds and reptiles [39–41]. Here, we highlight a remarkable example of convergent evolution whereby *CYP2J19* would have been independently recruited in the bird and turtle lineages, through changes in gene expression, to function in ketocarotenoid-based coloration from an ancestral retina-specific function. The ecological driver for this convergence in many instances may have been sexual selection [20,42].

Our results imply that red retinal oil droplets themselves were present in the common ancestor of the archelosaurs (archosaurs and turtles) [31], so that red oil droplets had a single origin in tetrapods (figure 3). An alternative would be if *CYP2J19* originally arose for a different function than red oil droplets, but this is unlikely because no other functions for ketocarotenoids have been described. The adaptive function of red retinal oil droplets in these early reptiles is an interesting and open question. Their acquisition presumably led to improved colour discrimination at long wavelengths, particularly in bright light [7,8], but the ecological context for this is obscure.

The inferred timing of the gene duplication that produced *CYP2J19* after the split with lepidosaurs is concordant with the absence of red oil droplets in this clade. The apparent absence of *CYP2J19* in crocodilian genomes could be explained by the derived loss of all oil droplets from the retina that has occurred in crocodilians, possibly due to nocturnality [43], as in snakes where an ancestral period of nocturnality may have resulted in loss of retinal oil droplets [44,45]. Although we cannot completely rule out the presence of a divergent *CYP2J19* locus in crocodiles and/or lepidosaurs, which was not identified in our genome searches, it is notable that our methods did successfully identify one *CYP2J* locus, as well as *CYP2D6* and *CYP2R1* in two crocodilian and two lepidosaur genomes (electronic supplementary material, figure S1). The function of *CYP2J40* is unknown, but the observed expression among all the tissues studied (in both turtles and birds [11]) does not suggest any direct involvement of this locus in red coloration.

As birds are phylogenetically nested within the saurischian dinosaurs [46], the continuous presence of red retinal oil droplets in the avian lineage since the split with Testudines implies the presence of red oil droplets in the retina of dinosaurs and other lineages (including pterosaurs) that are more closely related to the living birds than crocodiles. Building on the previous inference of colour vision in dinosaurs based on four cone opsin types [47], the presence of red retinal oil droplets in dinosaurs suggests that they possessed similar tetrachromacy to the birds and turtles, with good colour discrimination at long wavelengths. This is concordant with the proposed use of coloration in intraspecific signalling in dinosaurs, where putative melanosomes have been used to infer melanin-based coloration and iridescence [48–51]. The co-option of *CYP2J19* for red ketocarotenoid coloration in dinosaurs, as has occurred in birds and turtles, may also have been possible.

The implied presence of a functional carotenoid ketolase in turtles, which are primarily aquatic, is interesting in view of the availability of ketocarotenoids in potential prey items in aquatic environments, e.g. crustaceans, which means that in principle turtles might have lost ketolase activity. If true, this may be a legacy of their terrestrial ancestry in basal archelosaurs. Another possibility is that tight control of carotenoid concentration in retinal oil droplets is required for good colour vision, and that there is too much variation in dietary ketocarotenoids to rely on this source. In this context, it is interesting to note that

whereas, as expected, severe dietary carotenoid deprivation is harmful to colour vision in birds, dietary supplementation of carotenoids does not affect the carotenoid content in oil droplets used for colour vision [52–54].

In birds, the anatomical site of ketolation for red coloration has long been contentious but the pattern of *CYP2J19* expression has now shown that it is variable among taxa. Some species, e.g. zebra finch, perform ketolation in the peripheral tissues such as the beak where ketocarotenoids are deposited [11,55], whereas in other species such as ploceids, ketolation occurs centrally in the liver, and ketocarotenoids are transported in blood to sites of deposition (H Twyman, M Prager, NI Mundy, S Andersson 2016, unpublished data). In turtles, the presence of *CYP2J19* expression in red integument, but not in the liver, is indicative of the peripheral conversion model in this group.

Apart from birds and turtles, the only other vertebrate in which red retinal oil droplets have been reported is the lungfish *Neoceratodus* [56]. Microspectrophotometry measurements on these red droplets showed that they had similar spectral properties to the red droplets of birds [57]. However, acquisition of red oil droplets in this lineage was probably independent of that of archelosaurs because *CYP2J19* arose a long time after the split between lungfish and tetrapods, and this is supported by our failure to find a *CYP2J19-like* sequence in the *Neoceratodus* genome using BLAST searches.

In summary, we have uncovered a remarkable case where a gene with a strongly conserved function in colour vision has been independently co-opted for red ketocarotenoid-based integumentary coloration in turtles and birds via changes in patterns of gene expression. As *CYP2J19* arose within reptile evolution, the genetic basis for red ketocarotenoid coloration in amphibians and ray-finned fish warrants further research.

4. Material and methods

(a) Data mining and phylogenetic analysis of amniote *CYP2J* loci

BLASTn searches were conducted in all available reptilian genomes, belonging to the testudine (4), crocodilian (4) and lepidosaur (9) lineages (electronic supplementary material, table S1). We also performed BLASTn searches in the common ostrich (*Struthio camelus*) (Palaeognathae), which together with chicken (Galloanserae) and zebra finch (Neoaves) represent all three major extant avian lineages. Searches were conducted using *CYP2J*, *CYP2R1* and *CYP2D6* sequences taken from five Ensembl release 83 genomes: zebra finch (*Taeniopygia guttata*), chicken (*Gallus gallus*), human (*Homo sapiens*), anole lizard (*Anolis carolinensis*) and Chinese soft-shell turtle (*Pelodiscus sinensis*) [58]. Nomenclature for avian and reptile *CYP2J* genes was taken from a study on avian *CYPs* [59]. Previous work has shown that there are two lineages of avian *CYP2J* loci, *CYP2J19* and *CYP2J40* (31), whereas humans and other mammals have a single *CYP2J* locus, *CYP2J2*. *CYP2R1* and *CYP2D6* are single-copy *CYP* families that are closely related to *CYP2J* and were used as outgroups (31).

Nucleotide sequences were aligned in MEGA v. 6 [60] using MUSCLE [61]. Phylogenetic reconstructions of protein sequences derived from the nucleotide alignment were performed by maximum-likelihood in PhyML-SMS (Smart Model Selection), using model selection based on Bayesian information criterion (http://www.ebi.ac.uk/Tools/sfc/emboss_seqret/) [62]. The selected model was JTT + G6 (fixed at 1.997) + I (fixed at 0.089). Branch support was assessed with 1000 bootstrap pseudo-replicates.

(b) Laboratory methods

Captive-bred male ($N = 3$) and female ($N = 3$) three-month-old hatchlings of the western painted turtle were obtained by artificial incubation of eggs at constant temperatures that produce a single sex (26°C and 31°C, respectively), as previously described [63]. At this life stage, red coloration is present in the plastron but yet to develop on the neck. Individuals were euthanized by an overdose of the anaesthetic propofol, and stored in RNA*later*. The following tissues were studied: eye, neck skin, neck muscle, plastron, tail skin and liver.

Total RNA was extracted using QIAGEN RNeasy Mini kits. Separate extractions were performed on red and black regions of the plastron, yellow and black regions of the neck, and grey tail skin. Dissected tissues were individually placed in 1.5 ml Eppendorf tubes and submerged in liquid nitrogen before manual homogenization using an Eppendorf homogenizer and addition of Buffer RLT. The lysate was centrifuged for 2 min at 13 000 rpm in QIAshredder spin columns before proceeding with subsequent full-speed centrifugation step for 3 min. DNase digestion was performed using the RNase-Free DNase Set. All primers were designed in Primer3Plus using the western painted turtle genome [29].

First strand synthesis was performed with 10 µl total RNA and N_6 primer (0.5 µM) using SuperScriptII RT (Life technology Invitrogen) according to the manufacturer's instructions. RT-PCR reactions contained 1 × NH_4 buffer, $MgCl_2$ (1.5 mM), each dNTP (2.5 mM), each primer (0.4 µM), BioTaq DNA polymerase (Bioline) (0.5U) and cDNA (approx. 50 ng). Reactions were run in a G-Storm GS1 Thermal Cycler (Life Science Research) under the following conditions: 2 min at 94°C followed by 35–50 cycles of heating for 30 s at 94°C, 45 s at 60°C and 90 s at 72°C with a final extension of 5 min at 72°C. The amplified full-length fragment was purified using ExoSap-IT (Affymetrix) and sequenced on both strands via Sanger sequencing (electronic supplementary material) to confirm gene identity.

Quantitative real-time RT-PCR was carried out in an MJ Opticon2 (Research Engines) thermal cycler using the Quantitech SYBRGreen kit (Qiagen) for male and female retina, liver, red plastron, black plastron and yellow neck regions. We used three technical replicates for each condition, and three reference loci (*TBP*, *GAPDH* and *HPRT1*). The geNorm application for the evaluation of expression stability in the control genes was applied to assess the suitability of the reference loci [64]. *M*-values (denoted as the average pairwise variation of a control gene with all other control genes) for *TBP*, *GAPDH* and *HPRT1* were 1.051, 1.085 and 0.991, respectively, indicating suitability for their use. Differences in gene expression among tissues were assessed via analysis of variance using the 'car' package in RStudio v. 3.2 [65,66]. The Box-Cox power transformation for normality was applied, and lambda was fixed at 0.3 for subsequent statistical analysis. The assumptions of ANOVA were verified (electronic supplementary material).

Ethics. All procedures were approved by the IACUC of Iowa State University.

Data accessibility. DNA sequences: GenBank accessions KX553993-4.

Authors' contributions. H.T. designed the experiments, carried out molecular laboratory work, analysed the data and drafted the manuscript; N.V. and R.L. supplied the samples and helped edit the manuscript; S.A. helped to conceive the study and helped edit the manuscript; N.I.M. conceived the study, designed the experiments and drafted the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

Funding. We thank the BBSRC Doctoral Training Partnerships (to H.T.) for funding.

Acknowledgements. We thank Jason Head for helpful comments on the manuscript.

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