GTP cyclohydrolase 1 and axanthism in ball pythons: a new vertebrate model for pterin-based pigmentation

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Abstract:

Pterin (also known as pteridine) pigments are responsible for many of the bright colours observed across the animal kingdom. Yet, unlike melanin, the genetics of pterin-based pigmentation has received relatively little attention in animal colouration studies. Here, we use a lineage of axanthic ball pythons (*Python regius*) found in captivity as a model system for studying pterin pigmentation in vertebrates. We crowd-sourced ball python shed skin from commercial breeders and applied a case-control study design. Using whole-genome pool sequencing (pool-seq) and variant annotation, we found a premature stop codon in *GTP cyclohydrolase 1* (*gch1*) associated with the axanthic phenotype. The enzyme GCH1 plays a key role in pterin pigment biosynthesis as well as neurochemistry, catalyzing the first rate-limiting step of tetrahydrobiopterin (BH4) biosynthesis. Our work provides the first identification of an axanthism-associated gene in vertebrates and highlights the utility of ball pythons as a model system to study pterin-based pigmentation.

INTRODUCTION

Animal pigmentation has served as a valuable model for gaining insight into genetics, development, and evolution (Cott 1940; Kronforst et al. 2012; Caro 2017; Endler et al. 2017; San-Jose et al. 2017). Vertebrate pigmentation arises from the differential absorption of light by pigments within cells known as melanophores (melanocytes in endothermic vertebrates) and xanthophores. All vertebrates, from fish to mammals, produce a brown/black form of melanin derived from the amino acid tyrosine (Wakamatsu and Ito 2021). Additionally, poikilothermic vertebrates (fish, amphibians, and reptiles) display bright colouration through yellow/red pigments found in cells called xanthophores. Xanthophores get their vivid yellow or red colouration from pterin or carotenoid pigments. Whereas vertebrates can only obtain carotenoids from their diet (Maoka 2020), pterin pigments are synthesized *de novo* from precursor molecules such as guanosine triphosphate (GTP). Colouration can also be structural. Poikilothermic vertebrates use guanine nanocrystals in cells called iridophores to generate structural colouration through the constructive interference of light. Although melanin-based pigmentation has been extensively studied, these other pigments classes have received less attention.

The mouse has been central in studies of melanocyte biology, having been the primary model for discovering the genes responsible for melanocyte development: *Kit*, *Pax3*, *Mitf*, *Edn3*, *Ednrb*, and *Sox10* (Steingrímsson et al. 2005); melanin synthesis: *Tyr*, *Tyrp1*, *Dct* (McNamara et al. 2021; Wakamatsu and Ito 2021); and its regulation, like *Mc1r* (Garcia-Borron et al. 2005). Meanwhile, the zebrafish model has been instrumental for laying the

58 foundations of poikilothermic vertebrate pattern formation (Singh and Nüsslein-Volhard 2015).
59 Studies on zebrafish have shown that pattern formation arises locally from short- and long-
60 range cell-cell interactions between chromatophore types (Frohnhöfer et al. 2013). For
61 example, mutants lacking any one of the chromatophores (iridophores, melanophores, and
62 xanthophores) fail to develop stripes, often developing spots instead. In double mutants,
63 zebrafish lacking two of the three chromatophore types, the remaining chromatophore covers
64 the body uniformly (Frohnhöfer et al. 2013; Owen et al. 2020). However, how widely the details
65 of zebrafish cell-cell interactions apply to other vertebrate taxa is not known.

66 First discovered in the wings of butterflies (Hopkins 1895), pterin-based pigmentation
67 has received less attention in pigmentation studies despite being ubiquitous in nature (Andrade
68 and Carneiro 2021). The pterin pigment synthesis pathway has been discovered, in large part,
69 from work on *Drosophila* (Glassman and Mitchel 1959; Courtright 1967; Ziegler and Harmsen
70 1970; Goto and Sugiura 1971; Evans and Howells 1978; Spradling and Rubin 1983; Reaume et
71 al. 1991). Indeed, the first *Drosophila* mutant described by Thomas Hunt Morgan, *white*, lacked
72 eye pterin (drosopterin) and ommochrome pigments (Morgan 1910). Whereas melanin
73 pigmentation is often associated with adaptive phenotypes, like cryptic colouration (Barrett et
74 al. 2019; Harris et al. 2020; Baltazar-Soares et al. 2024), pterin pigments have been identified as
75 playing key roles in advertising within and between species (Kikuchi et al. 2014; Andrade and
76 Carneiro 2021). In vertebrates, zebrafish has been the primary model used to study
77 xanthophore biology, primarily xanthophore specification from neural crest cells (Kelsh et al.
78 1996; Odenthal et al. 1996; Parichy et al. 2000; Minchin and Hughes 2008) but also pigment
79 synthesis (e.g., Ziegler 2003; Lister 2019).

80 One way to gain insight into the genetics of bright colouration is by studying mutants
81 lacking a pigment, as first demonstrated by Thomas Hunt Morgan using fruit flies. Axanthic
82 animals are mutants that lack yellow and red pigments, yet contain melanin. Vertebrates with
83 this colour anomaly are rare. To our knowledge, only a few axanthic amphibians and reptiles
84 have been discovered in nature (Bechtel 1991; Jablonski et al. 2014; Cattaneo 2015; Kolenda et
85 al. 2017; Cavalcante and Bruni 2018; Borteiro et al. 2021; Schluckebier et al. 2022). Axanthic
86 vertebrate models are also rare in pigmentation research. A potential exception is the axanthic
87 colour morph in laboratory axolotls, first described by Lyerla and Dalton (1971). Axanthic
88 axolotls lack xanthophores, but they also do not have iridophores. Thus, axanthism in axolotls
89 acts at the level of chromatophore development rather than pigment synthesis. Axanthic
90 axolotls also have low viability, which can impact various aspects of research, including
91 breeding programs, population genetics studies, and comparative analyses.

92 Here, we use a line of axanthic ball pythons to study the genetics of yellow pigmentation
93 in a reptile model. We hypothesize that, assuming a similar mechanism of vertebrate pattern
94 formation as zebrafish (Frohnhöfer 2013; Singh and Nüsslein-Volhard 2015), axanthism in ball
95 pythons acts at the level of pterin pigment synthesis (Ziegler 2003), as opposed to carotenoid
96 metabolism or xanthophore development. We expect xanthophore development to be
97 unaffected because axanthic ball pythons exhibit wild-type melanin patterning, which is
98 consistent with intact cell-cell interactions. We also expect yellow pigmentation (or lack
99 thereof) to be associated with pterin-based pigmentation rather than carotenoids. Our
100 reasoning for this last expectation is that wild type ball pythons are born with yellow
101 colouration, before they have a chance to acquire exogenous carotenoids (e.g., zeaxanthin) and

are unlikely to obtain them from the egg. Snakes, being primarily carnivorous, likely have a diet poor in carotenoids, which are commonly found in plant matter, although some prey can contain carotenoids (e.g., birds).

METHODS

Sample collection, study design, DNA extraction, and sequencing

We crowd-sourced ball python shed skin from breeders, including five samples with the recessive VPI Axanthic colour morph (Table S1). The term VPI stands for Vida Preciosa International, and it is the name of the reptile-propagation company that received the first wild-caught axanthic snake from western Africa and propagated the phenotype in captivity. All VPI Axanthic (hereafter referred to as 'axanthic') ball pythons descend from this individual. We also received 38 samples for the recessive 'Clown' phenotype (Table S2), which we used as a control to look for candidate mutations for axanthic. Samples from other colour morphs ('Spider', n=26, Table S3; 'Enchi', n=7, Table S4; 'Ivory', n=15, Table S5; 'Lavender', n=14, Table S6) were collected as additional control sets. We used these controls because according to breeder's pedigree, they contained no axanthism variation. Similarly, the axanthic samples contained no Clown, Spider, Enchi, Ivory, or Lavender variation (i.e., inferred homozygous for reference alleles at these loci). Thus, we looked for candidate mutations by comparing genetic variation from the set of axanthic samples (i.e., pool) with multiple sets of reference samples known through pedigree analysis to not contain the axanthic variant.

The wildtype ball python colour morph exhibits black background with tan-coloured lateral saddles, with traces of yellow pigmentation (Figure 1A). A comparison of the wild type

123 and albino colour morph helps to show the normal xanthophore pigmentation and patterning:
124 for non-axanthics, yellow pigment tends to overlap with the tan coloured saddles and is missing
125 from the dark melanic regions (Figure 1B). In contrast, axanthic ball pythons lack yellow
126 pigmentation but retain a wildtype melanin pattern (Figure 1C).

127 We extracted and sequenced DNA from shed skins by following a standard three-day
128 phenol-chloroform procedure. DNA quantification and quality-checks were done on an Infinite®
129 200 Nanoquant (Tecan Group Ltd. Männedorf, Switzerland). We normalized DNA from each
130 sample and combined it into pools in equimolar amounts. We sent our pooled samples (i.e.,
131 case and controls) to the McGill Genome Centre for whole-genome PCR-based library
132 preparation and sequencing of 150 bp paired-end reads on one lane of the Illumina NovaSeq
133 6000 platform.

134 *Bioinformatics*

135 We used the *trim-fastq.pl* program of *Popoolation* (Kofler et al. 2011a) to filter raw reads based
136 on quality (--quality-threshold 20) and length (--min-length 50) parameters. This step filters
137 each read for a minimum quality score of 20 and minimum length of 50 bp. We used the
138 program *bwa-mem* with default parameters (Li and Durbin 2009) for read mapping along a
139 reference genome. Lacking a ball python reference genome, we aligned filtered reads to both
140 the draft Burmese python reference genome Pmo2.0 (Castoe et al. 2013) and the more recent
141 (unannotated) chromosome-length Burmese python reference assembly
142 *Python_molurus_bivittatus-5.0.2_HiC* (available at dnazoo.org). This chromosome-length
143 assembly is based on Hi-C data (Dudchenko et al. 2017; Dudchenko et al. 2018) aligned to the

144 annotated draft genome (Castoe et al. 2013) and has been used in previous studies to identify
145 colouration loci (Garcia-Elfring et al. 2023). We used *SAMtools* for SAM to BAM file format
146 conversion (Li et al. 2009) and removed reads with mapping quality below 20 (-q 20). *SAMtools*
147 and *Popoolation2* (Kofler et al. 2011b) were subsequently used to produce mpileup and sync
148 formats, respectively, prior to variant calling. We used *bcftools* to call and filter (*vcfutils.pl*
149 *varFilter*) variants for a minimum depth of five (-d 5) and mapping quality of 20 (-Q 20). SNP-
150 specific F_{ST} estimates were obtained using the program *fst-sliding.pl* of *Popoolation2* (Kofler et
151 al. 2011b). We generated F_{ST} values for SNP variants of VPI Axanthic (n=5) relative to the control
152 pools. Because the control (e.g., Clown, Spider, etc.) pools has no axanthic variation, we expect
153 the derived allele to be fixed in the axanthic data and the reference allele to be fixed in the
154 control pool. We thus used a F_{ST} threshold of 1.0 to identify candidate mutations. To confirm
155 the genomic region and candidate mutation of interest, we generated F_{ST} values between
156 axanthic data and additional control pools (Spider, n=26; Enchi, n=7; Ivory, n=15; Lavender,
157 n=14).

158 *Variant annotation and identification of polymorphisms and fixed differences*

159 We used the software *SnpEff* (Cingolani et al. 2012) to annotate variants mapped to the draft
160 and chromosome-length assembly. *SnpEff* uses a genome assembly and the corresponding
161 general feature format (GFF3) file to provide information about the functional impact of the
162 variants on genomic features. Within genomic regions showing elevated F_{ST} ('F_{ST} peaks'),
163 individual variants with an F_{ST} of 1.0, mapped to a protein-coding region, and expected to affect
164 gene product (e.g., nonsense mutations and small indels – 'high impact'; missense mutations –

165 ‘moderate impact’) were considered candidate mutations. We used the program *Protter*
166 (Omasits et al. 2016) to visualize the position and effect of a candidate mutation on the protein.
167 The candidate variant was confirmed with PCR amplification and Sanger sequencing the locus
168 using samples not included in the pooled libraries. We validated candidate loci by Sanger
169 sequencing samples with genotypes inferred from breeder pedigrees. We sequenced inferred
170 heterozygotes (n=10), homozygotes for the derived axanthism-causing allele (i.e., axanthic
171 individuals, n=6), and inferred homozygotes for the wild type allele (n=10). Primers used to
172 amplify the region with the candidate locus were designed with *Primer3* (Kõressaar and Remm
173 2007; Kõressaar et al. 2018). We used the following primers for PCR amplification: Forward: 5’-
174 GAAACTTTTGACCAGACTCAGTC, Reverse: 5’- CTTCAATCACCACAGCCACTCC.

175

176 **RESULTS**

177 Our whole-genome sequencing and variant calling identified 26,449,779 SNPs across all samples
178 received from ball python breeders. Analysis of allele frequency differences between the
179 axanthic pool and the control pool (Clown colour morph) demonstrates two areas of high
180 differentiation. One region (~24.8 Mb) on chromosome two (Figure 2A) and a second peak on
181 chromosome three, corresponding to the region containing the Clown mutation (Garcia-Elfring
182 et al. *in prep*). Comparing the axanthic data with additional control pools (Spider, n=26; Enchi,
183 n=7; Ivory, n=15; Lavender, n=14) confirms that the region of high differentiation on
184 chromosome two is associated with axanthism (Figure S1). Within the peak on chromosome
185 two, one variant had both high levels of differentiation (F_{ST} = 1) and is predicted to have a high

186 impact on the gene product. This variant is a nonsense mutation, and it maps to exon 5 of the
187 gene *gtp cyclohydrolase 1* (*gch1*), annotated as LOC103050242 in the draft assembly. The
188 candidate SNP consists of a C to T nucleotide change (c.520C>T), changing an arginine residue
189 (CGA) to a TGA stop codon (p.Arg174*). This genetic variant is expected to result in a truncated
190 protein (Figure 2B) that likely has essential Zn^{2+} binding domains missing. We validated the
191 axanthic variant found in poolseq data by PCR amplification and Sanger sequencing of the locus
192 of interest in additional individuals (inferred heterozygotes: n=10; homozygotes for the derived
193 axanthism-causing allele: n=6; inferred homozygotes for the wild type allele: n=10). All
194 individuals tested had the genotype that was inferred from pedigrees by breeders.

195

196 **DISCUSSION**

197 Captive-bred ball pythons have emerged as a promising model system in pigmentation biology,
198 offering unique opportunities to delve into the genetics of coloration in reptiles (Brown et al.,
199 2022; Dao et al., 2023; Garcia-Elfring et al., 2023; Lederer et al., 2023). In this study, we focused
200 on one of the axanthic lines of ball pythons to explore the genetic underpinnings of yellow
201 pigmentation in these snakes. Whereas yellow and red hues in the vertebrate integument can
202 stem from dietary carotenoids or endogenous pterin pigments, the carnivorous diet of snakess,
203 coupled with the yellow coloration with which wildtype ball pythons are born, led us to
204 hypothesize that pterin pigments serve as the primary basis for the yellow colouration in this
205 species. Additionally, considering the wildtype melanin pattern observed in axanthic ball
206 pythons, we postulated that any candidate mutation responsible for this trait would likely be

207 found in a gene associated with pterin pigment synthesis, rather than in a gene regulating
208 xanthophore cell-fate specification.

209 Consistent with our expectations, we found a perfect association between axanthism
210 and a premature stop codon in *gch1*, a gene coding a zinc-dependant enzyme (Auerbach et al.
211 2000) responsible for catalyzing the first rate-limiting step of the biopterin (i.e., pterin pigment
212 synthesis) pathway. Our work indicates that a disruption of *de novo* BH4 biosynthesis likely
213 results in the loss of yellow pigment colouration in axanthic ball pythons. The biopterin
214 biosynthetic pathway consists of three steps that convert GTP into tetrahydrobiopterin (BH4)
215 using the enzymes GTP cyclohydrolase I (coded by the gene *gch1*), 6-pyruvoyl-tetrahydropterin
216 synthase (*pts*), and sepiapterin reductase (*spr*). In mammals, this pathway is required for amino
217 acid metabolism and neurotransmitter (dopamine, serotonin) biosynthesis (Longo 2009). In ball
218 pythons, a mutation changed a susceptible CGA arginine codon (Romanov and Sukhoverov
219 2017) to a TGA termination codon, which is expected to truncate the protein and disrupt its
220 normal function, the effects of which manifest as axanthism in this lineage (VPI) of the colour
221 anomaly.

222 The biopterin pathway is shared across vertebrates and more divergent taxa. For
223 example, drosopterins cause red coloration in *Drosophila* eyes and lizard skin and are
224 derivatives of BH4, like all pterin pigments (Kim et al. 2013; Andrade et al. 2019). Studies of
225 xanthophore pigmentation in reptiles have attributed red colouration to pterins and yellow
226 colouration to carotenoids (Fitze et al. 2009; Haisten et al. 2015; Andrade et al. 2019; Andrade
227 and Carneiro 2021), although there are exceptions (Morrison et al. 1995). Which pigments are

228 primarily used for bright colouration, carotenoids or pteridines, can be the result of carotenoid
229 availability in the environment/diet (Stuart-Fox et al. 2021). Snakes are primarily carnivorous
230 and tend to have a diet low in carotenoids. Therefore, in snakes, yellow and red colouration is
231 probably the result of endogenous pterins rather than carotenoid pigment, as suggested by our
232 results.

233 For example, the red banding of the eastern coral snake and its mimic, the scarlet
234 kingsnake, is associated with drosopterin. However, the yellow colour is neither caused by
235 sepiapterin nor xanthopterin (Kikuchi and Pfennig 2012), the two yellow pterin pigments. One
236 hypothesis is that in many snakes yellow is produced by isoxanthopterin. Isoxanthopterin is
237 generally expected to give a white hue (Andrade and Carneiro 2021) due to its ultraviolet
238 absorption spectra but may experience a shift in absorbance toward the visual spectrum under
239 *in vivo* conditions, like lower pH (Kikuchi and Pfennig 2012; Kikuchi et al. 2014). Our results
240 implicate endogenous pterin pigmentation, not dietary carotenoids, in yellow colouration in
241 ball pythons. However, further work is needed to pinpoint the specific pterin pigment, or
242 pigments, involved.

243 In humans and mice, a functioning GTP cyclohydrolase I (coded by *gch1*) is required for
244 normal neurochemistry. GTP cyclohydrolase is a crucial cofactor in the metabolism of amino
245 acids tyrosine, phenylalanine, and tryptophan, as well as the synthesis of neurotransmitters
246 dopamine and serotonin (Nagatsu and Ichinose 1996; Kapatos et al 1999; Kapatos 2013).
247 Mutations associated with BH4 deficiencies have been linked to severe metabolic and
248 neurologic disorders (Nagatsu Ichinose 1996; Longo 2009; Fanet et al. 2021), like

249 phenylketonuria (Trujillano et al. 2017), hyperphenylalaninemia (Himmelreich et al. 2021),
250 DOPA-responsive dystonia (Hirano and Ueno 1999; Klein 2014; Yoshino et al. 2018; Kostić et al.
251 2020; Bradley et al. 2021), Parkinson's disease (Mencacci et al. 2014), and hereditary spastic
252 paraplegia (Varghaei et al. 2021). Indeed, GCH1 was discovered because of its association with
253 phenylketonuria (Kaufman 1963). Axanthism in ball pythons is not associated with movement
254 anomalies or neurological disorders (i.e., pleiotropy), unlike other colour morphs (e.g., Rose and
255 Williams 2014; Starck et al. 2022). Snakes, and perhaps reptiles in general, might have evolved
256 compensatory mechanisms or alternative pathways that mitigate the neurological effects of
257 *gch1* mutations, knowledge of which may lead to better therapeutic treatments.

258 The extensive phenotypic variation found in ball python colour morphs and the
259 different combinations of colour morphs that breeders have produced in captivity can be used
260 to shed light and test hypotheses about the mechanisms driving pattern formation. Recent
261 work indicates that all ball pythons lack one of the three chromatophore types, iridophores
262 (Tzika 2024). Ball pythons can therefore be thought of as natural analogs of *Shd* mutant
263 zebrafish that lack iridophores (Frohnhöfer et al. 2013). In addition to axanthism, ball python
264 colour morphs can exhibit xanthism, that is, increases in the brightness of yellow (which may in
265 fact reflect a change to melanin) or in the distribution of yellow pigment (e.g., the 'Enchi' colour
266 morph: Figure 1E). Indeed, many of the ball python colour morphs that seem to affect
267 melanophore migration (i.e., by showing altered melanin pattern: Tri-Stripe, Figure 1F) show
268 xanthophore pigmentation in the areas normally covered by dark melanin (Figure 1G). This
269 observation of non-overlap of black and yellow pigments suggests that xanthophores and
270 melanophores mutually repel each other, as seen in zebrafish (Frohnhöfer et al. 2013).

In zebrafish double mutants (*Shd*;*Pfe*), that is fish missing two of the three chromatophores (e.g., iridophores and xanthophores), the remaining chromatophore type (e.g., melanophores) covers the body uniformly, unimpeded by cell-cell interactions. As such, we expect the genetic basis of all-black melanic ball python morphs, like 'Cinnamon' (Figure 1H), to be caused by mutations to genes required for xanthophore development, allowing melanophores to spread to all areas of the body, as opposed to genes involved in melanin production, like *agouti*, as is commonly observed in mammals (e.g., Sasamori et al. 2017; Reissmann et al. 2020). Further evidence of a repulsive xanthophore-melanophore interaction comes from the Albino form of the Cinnamon morph (Figure 1I). Instead of observing an underlying pterin pigment pattern, which might be expected if melanism in ball pythons was caused by an over-production of melanin, the 'Cinnamon Albino' phenotype consists of an all-white ball python with no pterin pigmentation (Figure 1I).

Other recessive lines of axanthism are found in captive-bred ball pythons, including the line known as 'MJ'. VPI Axanthic and other lines of axanthism are not allelic with one another, meaning crosses between any two lines produce a double heterozygote with wildtype colour, potentially reflecting a different genetic basis for each lineage of axanthism. The different lines of axanthism can thus be used and combined with other colour variants (Figure 1D) to study in greater detail the pterin pigment biosynthesis pathway and discover new axanthism-causing genes in reptiles. Here, we showed with the VPI lineage that, consistent with expectations, pterin (not carotenoid) pigment metabolism is associated with yellow colouration in ball pythons.

CONCLUSION

We used ball python colour morphs to study the genetic basis of xanthophore pigmentation in snakes. We found axanthism is associated with a key gene in the biopterin biosynthesis pathway, providing the first evidence that pterins, not carotenoids, produce the yellow pigment observed in ball pythons. To our knowledge, *gch1* is the first axanthism-associated gene identified in vertebrates. Our results provide new research avenues for the study of axanthism and xanthophore pigmentation in snakes and highlights the utility of using captive-bred ball pythons in pigmentation research.

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Data accessibility

The data that support the findings of this manuscript will be uploaded to the NCBI Sequence Read Archive (SRA).

311 **Contributions**

312 AGE, APH, and RDHB conceived and designed the study. HR collected samples. AGE and JMA
313 performed the molecular work and AGE the bioinformatics and data analyses. HR performed
314 PCR and Sanger sequencing analysis. AGE wrote the manuscript with input from APH, HR, JMA,
315 and RDHB.

316

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Figure 1. Mendelian phenotypes (colour morphs) in ball pythons. Compared to the (A) wild type, (B) the albino colour morph lacks melanin pigment which allows the display of xanthophore (pterin) pigmentation and patterning, occupying the tan-coloured parts of the wild type. (C) The VPI Axanthic is characterized by a lack of yellow pigmentation on a wildtype pattern. (D) Combining colour morphs with axanthism (VPI Axanthic + Pastel + Fire is shown) can create different forms of the colour anomaly. (E) Some colour morphs like Enchi (Albino + Enchi is shown) are associated with changes in xanthophore migration (e.g., xanthism). (F) Tri-Stripe colour morph exhibits altered melanin patterning. (G) The Albino form of Tri-Stripe shows the effects of this colour morph on xanthophore pigmentation. (H) Melanic colour morphs like Cinnamon do not have xanthophore pigmentation, as shown by an (I) all-white snake in the Albino form. Photo credit: worldofballpythons.com

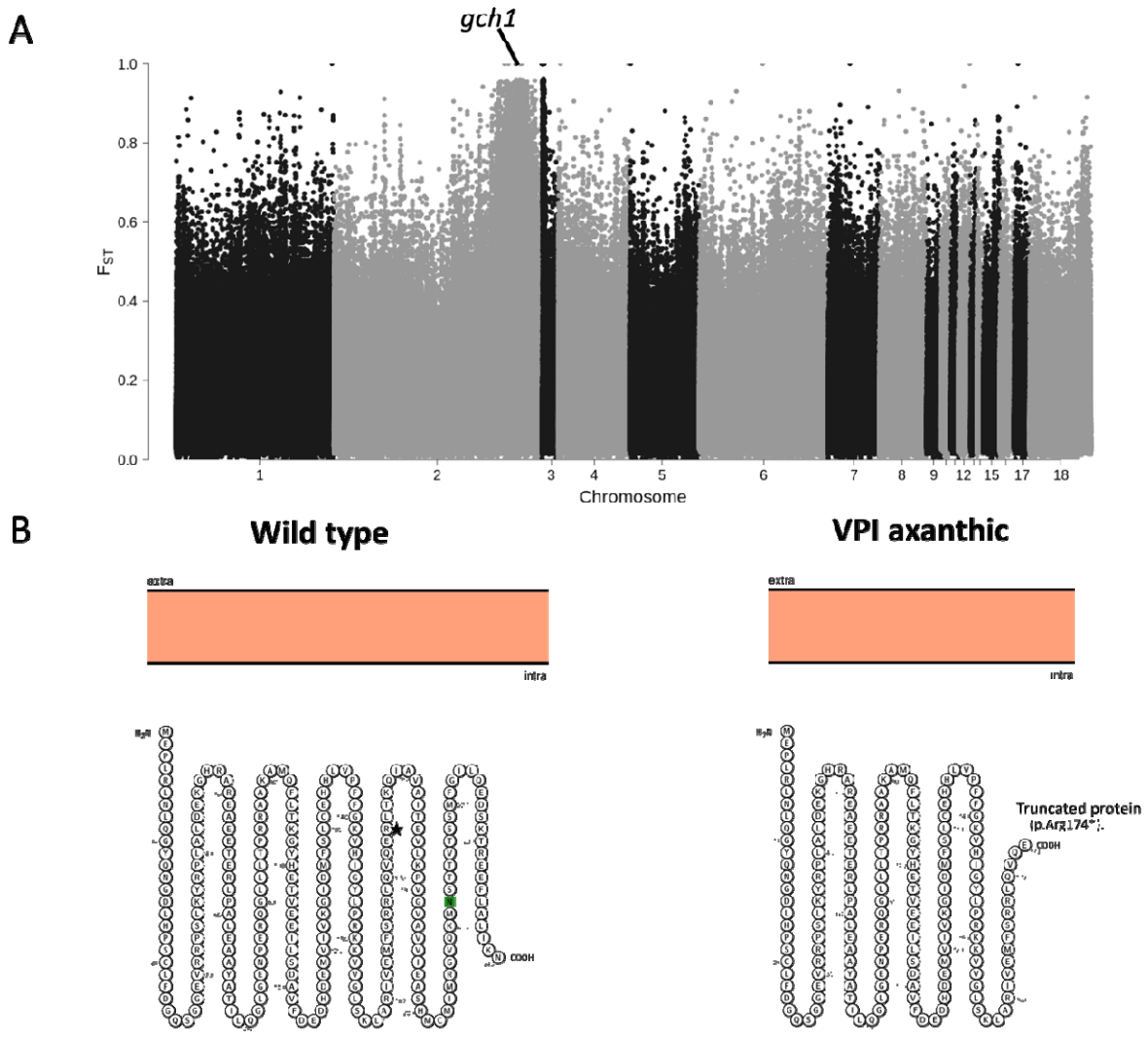

Figure 2. (A) Manhattan plot of F_{ST} values for SNPs mapped to the chromosome-length assembly. Genomic differentiation between VPI axanthic and a reference pool (Clown colour morph) shows a peak on chromosome two housing the axanthism candidate variant on *gch1*. (B) Burmese python amino acid sequence of *GCH1,* with the VPI Axanthic locus indicated by an asterisk (left panel). A nonsense mutation the on the 174th residue (p.Arg174*) likely leads to a defective truncated protein (right panel) and axanthism.