MOLECULAR CHARACTERIZATION AND EXPRESSION OF $G_{q/11}$

PROTEIN IN FISHES

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MOLECULAR CHARACTERIZATION AND EXPRESSION

OF $G_{q/11}$ PROTEIN IN FISHES

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ABSTRACT

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Teleost fishes and lower vertebrates adjust to changing light conditions by diurnal

movements of melanin pigment granules in their retinal pigment epithelium (RPE). RPE

is not directly activated by light and several observations suggest that some extracellular

signal from the retina may trigger the cascade of events leading to the granular movement

in RPE. Findings by González *et al.* (2004) and Phatarpekar *et al.* (2005) suggest that

pigment granule movements in fish RPE may be induced via G-protein coupled

muscarinic receptors. Based on these findings, I hypothesize that bluegill (*Lepomis

macrochirus*) RPE expresses at least one member of the $G_{q/11}$ family that mediates

the signal from the activated muscarinic receptor to the effector. To test this hypothesis, I characterized cDNAs encoding two G-proteins from the $G_{q/11}$ family. The subtype identity of these novel cDNAs and the encoded G-protein fragments from bluegill were examined using phylogenetic analyses. Their expression was examined in adult bluegill and zebrafish eyes using RT-PCR and immuno-histochemistry. Immunolabeling was also used to study the ontogeny of $G\alpha_{q/11}$ expression in zebrafish eyes. My results indicate that bluegill express both *bona fide* $G\alpha_q$ and a second member of the $G\alpha_{q/11}$ family, but that only $G\alpha_q$ is expressed in eye. These findings imply that $G\alpha_q$ could play a specific role in pigment movement in fish eyes. It also lays the groundwork for the future elucidation of the function of these genes in carbachol-induced pigment granule dispersion.

CHAPTER I

INTRODUCTION

Light adaptation is the ability of the visual system to adjust its sensitivity and performance to the ambient level of illumination. In a normal day-night cycle, the illumination on earth's surface varies by more than ten orders of magnitude, making light adaptation highly important for the survival of an organism. In vertebrate eyes, photo-adaptation occurs at several stages within the retinal network (Purpura *et al.*, 1990; Schmitt and Dowling, 1999). For example, in mammals and higher vertebrates, the amount of light reaching the retina is controlled by their adjustable pupil. In addition, within the photoreceptors, many mechanisms and several stages for adaptation have been reported (see Hood and Birch, 1993). For instance, nocturnal organisms like cats, mice and rats have a rod-dominant retina while some organisms like the lizards and ground squirrels have a cone-dominant retina (Farber *et al.*, 1981). In lower vertebrates like teleost fishes and amphibians that have a fixed pupil, light adaptation is achieved by retinomotor movement, a collective term used to describe movements of the rod and cone photoreceptors in the retina and the movement of melanin pigment granules in the retinal pigment epithelium (Douglas, 1982; Burnside and Nagle, 1983). Retinal pigment epithelium (RPE) is a monolayer of tissue located between the neural retina and choroid that functions in the physiological support of the retina (see Zinn and Marmor, 1979).

In teleost fishes, during light, the melanin pigment granules migrate from the cell body of the RPE into the apical projections that interdigitate with the rod photoreceptors (Figure 1a), shielding them from photobleaching (Douglas, 1982; Burnside and Nagle, 1983; King-Smith *et al*., 1997; García, 1998). Likewise, during light, the cones remain contracted and closer to incoming light than rods (Cavallaro and Burnside, 1988). In dark, the position of rod and cone photoreceptors are reversed, and the melanin pigment granules aggregate at the base of the RPE (Figure 1b) allowing the photoreceptors to maximize light capture (Burnside and Nagle, 1983; Cavallaro and Burnside, 1988; Iuvone *et al*., 2005).

Photoreceptors (rods and cones) respond directly to light and some endogenous circadian cues (McCormack and Burnside, 1991; Burnside, 2001). For example, serotonin (Zaunreiter *et al*., 1998b), melatonin (Zaunreiter *et al*., 1998a), and dopamine (Dearry and Burnside, 1989) have been linked to the retinal circadian clock in fishes. But RPE do not respond directly to light. In other words, RPE isolated from retina does not disperse pigment in response to light, nor does RPE with dispersed pigments aggregate pigment granules in the dark (Garcia and Burnside, 1994). Rather, pigment dispersion in RPE is presumed to be dependent on neurotransmitters and perhaps other small molecules diffusing from the neural retina (García and Burnside, 1994).

(a)   (b)
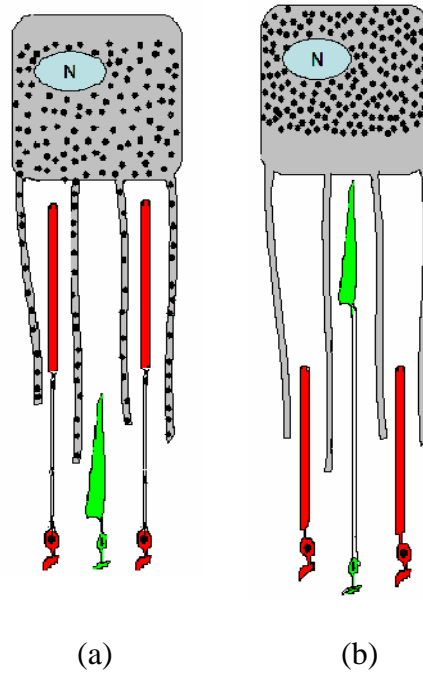
Figure 1. Pigment granule movements in fish retinal pigment epithelium. Cartoon depicting a cell of retinal pigment epithelium (a) during light, showing fully dispersed pigment granules with the rods (red) elongated and cone (green) contracted and (b) during dark when the pigment granules are aggregated, with the rods (red) contracted and cone (green) elongated between the apical processes.

Using pharmacological studies, Dearry and Burnside (1988) first reported that dopamine, acting via D2 receptors, may regulate retinomotor movements in green sunfish (*Lepomis cyanellus*) RPE, specifically by causing light-adaptive responses. Their studies also showed that dopamine induces light-adaptive cone contraction in isolated dark cultured retina and that when D2 receptors were blocked, cone contraction was also inhibited (Dearry and Burnside, 1986). Furthermore, dopaminergic cells were identified in the inner plexiform layer of retina by Mangel and Dowling (1987).

Later, it was shown that retinomotor movements persisted in fish whose dopaminergic cells had been ablated by treatment with 6-hydroxydopamine (Douglas *et al*., 1992; Ball *et al*., 1993). This suggested that neurochemicals in addition to dopamine may be involved in light-induced pigment granule dispersion. The evidence that the cholinergic agent, carbachol, can induce pigment granule dispersion (García, 1998) was the first study that raised the possibility of acetylcholine acting in addition to dopamine in regulating retinomotor movements. The idea of cholinergic signaling in light adaptation is relatively new and much less well derived. Studies have shown that flicker stimulation increases acetylcholine release and that the amount of release is proportional to flicker frequency (Vivas and Drujan, 1980). Furthermore, cholinergic cells have been identified in the amacrine cells of the retina (Arenzana *et al*., 2005). Thus, after numerous studies on retinomotor movements in the last two decades, a possibly new model of signal transduction has been suggested for light adaptation by González *et al*. (2004) and Phatarpekar *et al.* (2005). Their results suggest that light adaptive pigment granule dispersion in fish RPE may be elicited via specific G-protein coupled receptors called muscarinic receptors.

Muscarinic receptors are cholinergic, heptahelical receptors that are of five subtypes in mammals (M1-M5) (Caulfield and Birdsall, 1998), though only M2, M3 and M5 receptor subtypes have been described in fishes. Zebrafish has been shown to express both M2 and M5 muscarinic receptors (Hsieh and Liao, 2002) while only M3 subtype has been described in catfish melanophores (Hayashi and Fujii, 1994). Using molecular and pharmacological studies, Phatarpekar *et al*. (2005) characterized M2 and M5 receptor subtypes from bluegill cDNA and studied their expression in bluegill RPE and other tissues. Their results show that M5 but not M2 receptor subtype is expressed in bluegill RPE and hence is the more likely mediator of carbachol-induced pigment granule dispersion.

The model of signal transduction suggested by Phatarpekar *et al*. (2005) involves the activation of the M5 receptor by carbachol which triggers a member of the $G_{q/11}$ protein family involved in phosphoinositide hydrolysis, that further activates a cascade of events leading to pigment granule dispersion in RPE. Though this model is well supported by several studies, it is still in need of further testing to clarify the specific $G\alpha_{q/11}$-protein, its effector and further downstream events. Pharmacological agents like GPAnt-2a (Yajima *et al*., 1997) and YM-254890 (Takasaki *et al*., 2004) have been reported to be highly selective in blocking $G\alpha_{q/11}$ proteins. While these drugs are powerful tools to study $G\alpha_{q/11}$-mediated signaling, they have been characterized predominantly for mammals and may not be highly selective for fish. Furthermore, no pharmacological agents that are capable of selectively activating or blocking individual members of $G\alpha_{q/11}$ family have been identified thus far or made commercially available. So molecular characterization of $G\alpha_{q/11}$ subtypes and studies of their expression in fish

RPE might help resolve the step following muscarinic receptor activation in the model

suggested by Phatarpekar *et al*. (2005).

Findings by Phatarpekar *et al.* (2005) led me to hypothesize that $G\alpha_q$ and/or $G\alpha_{11}$

may be expressed in bluegill RPE and may have a specific role in the signaling pathway

that leads to pigment granule dispersion. The focus of my thesis research was to

molecularly characterize $G\alpha_q$ and $G\alpha_{11}$ in bluegill sunfish (*Lepomis macrochirus*) and to

study their expression in fish eyes using RT-PCR and immuno-histochemistry.

G-proteins are guanine nucleotide-binding, heterotrimeric, membrane-associated

proteins that function as molecular transducers, relaying signals from cell-surface

receptors to downstream effectors. They are composed of α (39-52 kDa), β (35-36 kDa)

and γ (8-10 kDa) subunits (Birnbaumer, 1990; Simon *et al*., 1991). They have a

conserved structure and share a common molecular switch mechanism mediated by the

binding and hydrolysis of GTP (Bourne *et al*., 1990). In resting state, the α-subunit is

bound to GDP and all three subunits remain associated as a trimer (Figure 2a). Activation

of the receptor by an agonist induces a shift in receptor conformation, triggering the G-

protein activation by replacement of GDP by GTP in the α-subunit and dissociation of the

α-subunit from the βγ dimer (Figure 2b). Both the α-subunit and the βγ dimer can then

interact independently with specific effectors (Gilman, 1987; Birnbaumer, 1990). The

hydrolysis of GTP to GDP causes the reassociation of the α-, β- and γ-subunits leading to

G-protein inactivation. As of 2003, 28α , 5β and 12γ subunits have been identified and

sequenced (Cabrera-Vera *et al*., 2003). Based on their α subunit sequence homology and

function, Gα-proteins are broadly divided into $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Simon *et al*.,

1991; Neer, 1995; Rens-Domiano and Hamm, 1995). The G-proteins under each family, their function and expression in various mammalian tissues are given in Table 1.

The $G\alpha_{q/11}$ class of G-proteins have been the focus of numerous studies not only due to their profound effects on the pathology of chronic inflammations overall (Das *et al.*, 2000), but also due to their role in heart disease (Webster and Bishopric, 2003), Alzheimer's disease (Kelly *et al.*, 2005), asthma (Gosens *et al.*, 2006), autism (Allgeier *et al.*, 1994), and many others. The four members of this family, namely $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15/16}$, are pertussis toxin-insensitive and are involved in phosphoinositide hydrolysis.
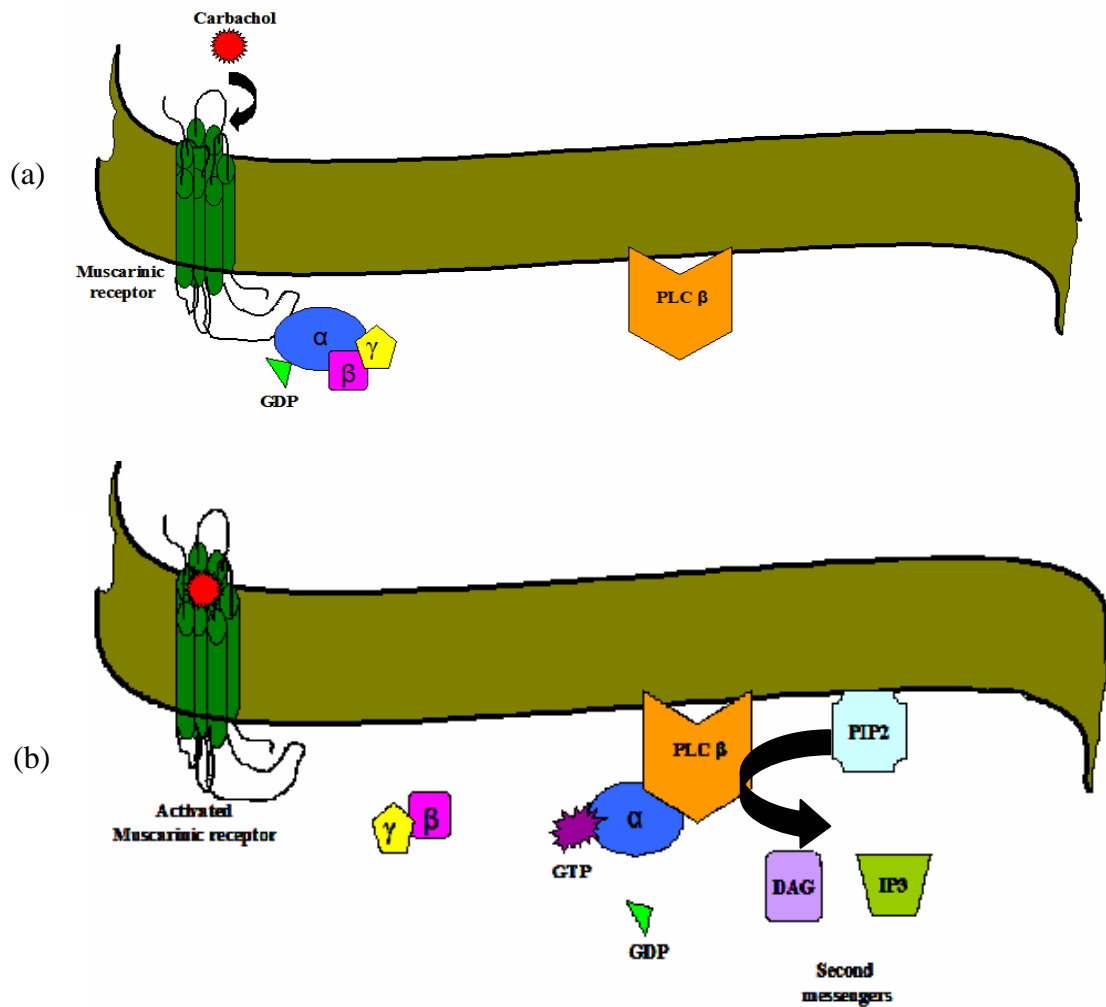
Figure 2. Model of Gα$_{q/11}$ protein activation. Diagram showing (a) Gα$_{q/11}$ in its resting state with all subunits associated as a trimer and (b) Gα$_{q/11}$ activation by replacement of GDP with GTP and dissociation from βγ dimer in response to ligand binding receptor. The activated GTP-associated Gα$_{q/11}$ activates phospholipase C-β (PLC β) which hydrolyzes phosphatidyl inositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG).

Table 1. Gα-protein classification, expression and their effectors in mammalian tissues. This table was adapted from a review by Wettschureck and Offermanns (2005).

G-protein family	Members	Expression	Effectors
Gα$_s$	Gα$_s$	Ubiquitous	▲ AC (all types)
	Gα$_{olf}$	Olfactory epithelium, brain	▲ AC
	Gαs$_{XL}$	Neuroendocrine	▲ AC
Gα$_i$	Gα$_{i1}$	Widely distributed	▼ AC(I, III, V, VI, VIII, IX)
	Gα$_{i2}$	Ubiquitous	▼ AC
	Gα$_{i3}$	Widely distributed	▼ AC
	[1]Gα$_{o1}$	Neuronal, neuroendocrine	▼ VDCC
	[2]Gα$_{o2}$	Neuronal, neuroendocrine	▼ VDCC
	[3]Gα$_{o3}$	Brain	Unclear
	Gα$_z$	Neuronal, platelets	▲ AC(V,VI), Rap1GAP
	Gα$_{gust(t3)}$	Taste cells, brush cells	▲ PDE
	Gα$_{t1}$	Rods, taste cells	▲ PDE 6 (γ-subunit)
	Gα$_{t2}$	Cone photoreceptors	▲ PDE 6 (γ-subunit)
Gα$_{q/11}$	Gα$_q$	Ubiquitous	▲ PLC β 1-4
	Gα$_{11}$	Almost ubiquitous	▲ PLC β 1-4
	Gα$_{14}$	Lung, kidney, testes, spleen	▲ PLC β 1-4
	[4,5]Gα$_{15/16}$	Hematopoietic cells, keratinocytes	▲ PLC β 1-4
Gα$_{12/13}$	Gα$_{12}$	Ubiquitous	PDZ-RhoGEF/LARG, Btk, Gaplm, cadherin
	Gα$_{13}$	Ubiquitous	P115RhoGEF, PDZ-RhoGEF/LARG, Radixin

[1,2,3]- Nürnberg et al., 1994; [4]-Taylor et al., 1991; [5]-Wu et al., 1992.
▲, activates; ▼, inhibits; AC, adenylyl cyclase; PDE, phosphodiesterase; PLC, phospholipase C; VDCC, voltage-dependent Ca^{2+} channel; RhoGEF, Rho guanine nucleotide exchange factor; Btk, Bruton's tyrosine kinase.

All four members activate phospholipase C-β isomers that stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), which yields inositol 1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG), both of which can act as intracellular second messengers (Wu *et al*., 1992; Broadley and Kelly, 2001). IP3 stimulates the

release of calcium by binding to receptors located on the endoplasmic reticulum. DAG

and an increase in intracellular calcium levels activate protein kinase C (Broadley and

Kelly, 2001). Calcium has many other effectors as well.

The sequence homology and expression of the members of $G\alpha_{q/11}$ family vary.

While $G\alpha_q$ and $G\alpha_{11}$ share 88% homology in mammals and are expressed ubiquitously

(Strathmann and Simon, 1990; Milligan and Mitchell, 1993), $G\alpha_{14}$ is 81% identical to $G\alpha_q$ and its expression is restricted to kidney, spleen, lung and testes in mammals (Wilkie *et al*., 1992). $G\alpha_{15}$ and its human counterpart $G\alpha_{16}$ are less than 60% homologous to $G\alpha_q$

and are restricted to hematopoietic tissue (Amatruda *et al*., 1991; Wilkie *et al*., 1992) and

keratinocytes in the skin (Rock *et al*., 1997) in mammals. Despite being members of the

$G\alpha_{q/11}$ family, $G\alpha_{14}$ and $G\alpha_{15/16}$ are not likely to be involved in light adaptive pigment

granule movements for two reasons.

Firstly, both members have restricted expression in specific tissues like spleen,

kidney, testes, lung and hematopoietic cells, respectively. Moreover, there has not been

any report about the expression of these subtypes in vertebrate eyes. Secondly, despite

their promiscuity in receptor coupling, neither $G\alpha_{14}$ nor $G\alpha_{16}$ has been shown to couple to

muscarinic receptors. While $G\alpha_{14}$ has been reported to couple to histamine receptors

(Kuhn *et al*., 1996) and, α_1-adrenergic and interleukin-8 receptors in transfected COS-7

cells (Wu *et al*., 1992), $G\alpha_{15/16}$ has been shown to be coupled to chemokine receptors for

interleukin 8, C5a, and fMLP (Offermanns and Simon, 1995), and histamine receptors

(Kuhn *et al*., 1996). But both $G\alpha_q$ and $G\alpha_{11}$ couple indiscriminately to a wide range of

receptors including muscarinic, histamine, parathyroid hormone, thyrotropin-releasing

hormone, gastrin-releasing peptide, vasopressin, gonadotrophin releasing hormone,

angiotensin II, bradykinin and α_1- adrenergic receptors (Shapira *et al*., 1998), and both members are ubiquitously expressed in mammalian tissues.

These observations raise the possibility that $G\alpha_{11}$ and/or $G\alpha_q$ may be involved in transmitting the signal from the muscarinic receptor to the effector. In addition, both members have played a role in light adaptation in many vertebrate and invertebrate models. For example, using immuolabelling and *in situ* hybridization techniques, some studies (Peng *et al*., 1997; Kasahara *et al*., 2002) showed that $G\alpha_{11}$ is expressed in chick photoreceptors and pinealocytes, and that its activation may play a role in light adaptive phase shifting in pinealocytes. Peng *et al.* (1997) showed $G\alpha_{11}$ immunoreactivity in the rod photoreceptors and $G\alpha_q$ immunoreactivity in the amacrine layer of bovine retina. Among invertebrates, $G\alpha_q$ expression has been documented in *Drosophila* (Strathmann and Simon, 1990), crayfish (Terakita *et al*., 1996) and in squid photoreceptors (Suzuki *et al*., 1995). Other studies have also shown the involvement of $G\alpha_o$-mediated signaling in invertebrate vision (Terakita *et al*., 1996).

In spite of the numerous studies on the signaling pathway of retinomotor movements in fish, little is known about the G-protein subtypes expressed in fish eyes or their role in light adaptation. To my knowledge, there is only one study (Sarwar *et al*., 1996) on G-protein subtypes expressed in fish. Sarwar *et al.* (1996) aligned Gα subunits from Japanese puffer fish (*Takifugu rubripes*) and compared their similarities with those of mammalian G-proteins. Though their study showed that *Takifugu* sequences were homologous to their mammalian orthologs, 90% of the amino acid sequences used in their study were partial (representing 30% of the expected sequence), and no phylogenetic inferences about relationships among G-protein subtypes were evaluated.

Similarly, though Gα proteins are expressed throughout the prenatal development of vertebrates, only a few studies have addressed the role of Gα-protein in vertebrate development (Wettschureck and Offermanns, 2005). Most insights into their role in development were from studies on mutant mice lacking specific Gα-subtypes. It was shown that null mutations of the genes coding for individual Gα-subtypes proved to be lethal to embryos (Offermanns *et al*., 1998), thus indicating their pivotal role in development. Another report by Shah and Hausman (1996) suggest the involvement of $G\alpha_{q/11}$ in retinal development in chick.

In this thesis, I report my results from examining the G-protein subtypes expressed in bluegill using a PCR approach, laying the groundwork for future elucidation of their role in carbachol-induced pigment granule dispersion. The subtype identity of one novel G-protein cDNA fragment ($G\alpha_q$) from bluegill was confirmed using several phylogenetic methods. Analysis of the second G-protein cDNA fragment failed to yield a definitive identity based on the current nomenclature, raising the possibility that a new subclass should be designated (e.g. G_{q1} and G_{q2} or G_{10}). The expression of these genes was examined in bluegill and zebrafish eyes using RT-PCR and immuno-histochemistry. Immunolabelling was also used to study the ontogeny of G-protein expression in zebrafish eyes. My results indicate that of the two G-proteins uncovered, only $G\alpha_q$ is expressed in retina and RPE. These findings suggest that $G\alpha_q$ could be involved in the signal transduction pathway linking muscarinic receptor activation to pigment granule dispersion.

CHAPTER II

MATERIALS AND METHODS

Fish maintenance

Bluegill (*Lepomis macrochirus*) and zebrafish (*Danio rerio*) were kept in aerated

55 gallon and 10 gallon aquaria, respectively, on a 12 hour light/12 hour dark cycle room

for at least two weeks prior to use. The protocols for animal use were approved by the

Texas State Animal Care and Use Committee (Protocol # 069744F82). Prior to killing,

the fish were anesthetized using tricaine methylsulfonate (Western Chemical Inc.,

Scottsdale, AZ).

Isolation of total RNA and generation of cDNA

Total RNA was isolated from 10 mg samples of bluegill brain, heart, retina and

RPE using an RNAqueous-4PCR kit (Ambion, Austin, TX), following the

manufacturer's instructions. For zebrafish, the RPE and retina were too difficult to

separate without contamination from other tissues and hence total RNA from whole eyes

was extracted. Briefly, the tissue was isolated and homogenized in RNase-free tubes

provided in the kit using guanidinium thiocyanate and passage through silica-based

buffer. Total RNA (8 μl) was then used to generate cDNA, using the RETROscript RT-

PCR kit (Ambion, Austin, TX), following the manufacturer's instructions. The two-step

protocol involved denaturing of total RNA and oligo (dT) primers at 82°C for 3 min
before adding remaining RT solutions, including RT buffer and reverse transcriptase.

PCR amplification of cDNA

The cDNA transcripts were amplified in a 50μl polymerase chain reaction. The
primers for the reaction were originally designed based on the alignment of vertebrate Gα
$_{11}$ and Gα$_q$ sequences. The complete coding sequences of Gα$_{11}$ and Gα$_q$ were downloaded
in FASTA format (Pearson, 1990) from GenBank (Bilofsky and Burks, 1988) database.
As no fish Gα$_{11}$ and Gα$_q$ sequences were available for alignment, chick Gα$_{11}$ and mouse
Gα$_q$ were used as query sequences to search the *Takifugu rubripes* genome available at
http://www.ncbi.nlm.nih.gov/BLAST/Genome/fugu.html using the Basic Local
Alignment Search Tool (BLAST) (Altschul *et al*., 1990). The putative *Takifugu*
sequences were then aligned with other vertebrate G-protein sequences using CLUSTAL
W program (Thompson *et al*., 1994). Highly homologous regions near the 5' and 3' ends
of the alignment were selected for designing primers. The forward and reverse primers
designed were about 18-23 bps long and their compatibility, G+C percent and self-
complementarity were checked using an oligonucleotide properties calculator available
online at http://www.basic.northwestern.edu/biotools/OligoCalc.html. The list of primers
used for amplifying putative Gα$_{11}$ and Gα$_q$ from bluegill brain cDNA are given in Table
2. The primers were purchased from BioSynthesis Inc, Lewisville, TX. The primers were
reconstituted in TE buffer (comprising 10 mM Tris pH 7.5 and 1mM EDTA) and were
diluted further in PCR-grade water before use.

PCR was carried out by adding 1 µl cDNA, 3 µl MgCl (25 mM), 10 µl 5X GoTaq

flexi buffer, 2 µl dNTPs (10 mM each), 0.5 µl GoTaq Flexi DNA polymerase (5 µ/µl)

(Promega Inc, WI), 1 µl each of the primers and 31.5 µl nuclease-free water and

subjecting the mixture to the following conditions: one cycle of initial denaturation at

94°C for 1 min 4 sec, 40 cycles each of denaturation at 94°C for 30 sec, annealing at

50°C for 30 sec and extension at 72°C for 1min 30 sec, and a cycle of final extension at

72°C for 5 min. The PCR products and 1kb GoTaq DNA ladder (Promega Inc, WI) were

loaded onto a 1% agarose gel inundated with TAE buffer (containing 0.4 M Tris, 0.01 M

EDTA and 0.2 M acetic acid, pH 8.5) and were subjected to electrophoresis at 120V for

1hr. To verify the presence and the size of the product, the gel was stained in ethidium

bromide for 5 min and observed under ultraviolet light after destaining in distilled water.

A photograph of the gel was obtained using Nikon COOLPIX 990 digital camera with a

UV filter, and the picture was processed with Adobe Photoshop Version 5.5 software

(Adobe Systems Inc., San Jose, CA). PCR products of approximately 1000 bp were

purified using High Pure PCR Product Purification Kit (Roche Diagnostics Corp, IN)

following the instruction manual, and sequenced using automated cycle sequencing

method at Retrogen Inc, CA. The sequences obtained were analyzed and edited

individually using the software Sequencher 4.2 (Gene Codes Inc., Ann Arbor, MI).

Table 2. Table showing oligonucleotide primers used for the isolation of Gα$_{11}$ and Gα$_q$ fragment from bluegill. The length of the primers, their percent GC content and melting temperatures are also provided.

Primer name	Primer sequence	Primer length	G+C content (%)	Melting temp (^{o}C)
G11F1	ATG ACT CTG GAG TCC ATG ATG G	22	50	66
G11F2	GAA TCA GAC AAC GAG AAC CGC A	22	50	66
G11F3	GTA CTC AGA CGA GGA TAA GAG	21	47.6	62
G11R1	TTG AGG TTG AGG ATG GTG TC	20	50	60
G11R2	GCT GTA GGA TGG TGT CTT TG	20	50	60
G11R3	GGC GGT CAT AGG CCT CTT GG	20	65	58
GqF	AGC GAG GAG GCC AAG GAA GC	20	65	66
GqR	TCC TCC ATT CGG TTC TCA TTG TCT	24	45.8	70
β-actin F	CTC CAT CAT GAA GTG CGA CGT	21	52	54
β-actin R	CAG ACG GAG TAT TTG CGC TCA	21	52	54

The identity of the sequenced genes were initially evaluated by comparing the obtained sequence with known nucleotide sequences in GenBank (Bilofsky and Burks, 1988) using the search tool BLASTn (nucleotide-nucleotide BLAST).This tool compares the query sequence against all available nucleotide sequences, and the percentage similarity between the top hits (best matches) and the query sequence was obtained. The nucleotide sequence was then translated into the corresponding amino acid sequence using the 'Translate tool' available at the proteomics website http://ca.expasy.org/tools/dna.html. The deduced amino acid sequence was used to search the available protein sequences in GenBank (Bilofsky and Burks, 1988) using BLASTp (protein-protein BLAST) tool. The percent similarity and percent identity of bluegill amino acid sequences with other vertebrate Gα$_q$ and Gα$_{11}$ sequences were also calculated

using BL2SEQ (Tatusova and Madden, 1999) available at NCBI

http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi.

Once the identity of the genes were tentatively confirmed using different search tools, the putative bluegill G-protein fragments were again aligned with their vertebrate orthologs and more outer primers (Table 2) were designed on the 5' and 3' ends to get the ends of the gene. All primers once designed, were checked for their compatibility using the oligonucleotide calculator and were purchased from BioSynthesis Inc, Lewisville, TX. The PCR products were sequenced each time and the final sequence fragments were edited and incorporated into the initial contiguous sequence using Sequencher 4.2 (Gene Codes Inc. Ann Arbor, MI) to form a contiguous sequence.

Phylogenetic analyses

In order determine the subtype identity of the sequenced fragments, complete coding sequences of forty-four G-protein α subunits were downloaded in FASTA format (Pearson, 1990) from GenBank (Bilofsky and Burks, 1988) (Table 3) and were aligned using Sequencher 4.2 (Gene Codes Inc., Ann Arbor, MI, USA). Different alignments were tried by first aligning Gα sequences of each subtype, followed by aligning each subtype with other subtypes. The criterion for optimal alignment was the perfect alignment of "landmarks" or highly conserved motifs in all G-protein sequences. The DNA sequences were translated into proteins in MacClade 4.05 OS X (Maddison and Maddison, 2001) using universal genetic code and for every gap in the protein sequence, a corresponding triplet gap was introduced manually in the aligned nucleotide sequence. The protein and DNA alignment files were executed in PAUP* (Swofford, 1998), and the

G-protein sequences from mold (*Mucor circinelloides*) and fungi (*Ustilago maydis*) were used as outgroups in all analyses. Before reconstructing phylogenies using different methods, the g1statistic was used to estimate the phylogenetic signal for the dataset by generating a frequency distribution of thousand random trees.

Tree topologies were obtained under parsimony criterion using heuristic search with five thousand random replications using stepwise additions for both the DNA and protein alignments. The third position codons were downweighted for the parsimony analysis as the nucleotides at the third codon positions evolve faster than those at the first and second codon positions. Bootstrap statistical support for individual clades was obtained by five thousand random replications with one stepwise addition using heuristic search. A neighbor joining tree was generated using distance as the optimality criterion. Tamura-Nei model, which assumes unequal base frequencies and three substitution rates, was used for distance correction and the rates for variable sites were assumed to be equal.

Modeltest 3.7 (Posada and Crandall, 1998) was used to select the model of evolution for Bayesian and maximum likelihood analysis using DNA alignment. The General time reversible + I (invariant sites) + G (gamma distribution) model was chosen using the Akiike criterion for having the highest log likelihood. Maximum likelihood was estimated in PAUP* using the parameter estimates obtained from Modeltest. Fifty bootstrap replications using one heuristic random stepwise addition was used to determine statistical support values. The Bayesian analysis with GTR+I+G as evolutionary model, was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). *Ustilago maydis*, a fungus, Gpa1was set as the outgroup for the analysis. An initial Markov Chain Monte Carlo search was set to run ten thousand generations with a sample

frequency of one hundred. The runs were repeated with increasing number of generations until the sum of the log likelihoods of trees converged to a stable value. Based on the number of generations taken to stabilize the log likelihood value, a final run was set, with the number of generations set to a million with a sample frequency of hundred. The burnin, which represents the number of trees that would be ignored while the consensus tree is created, was set to twenty five percent of the number of trees sampled (2500). The tree file produced in MrBayes was opened in PAUP*, and a consensus tree was generated.

Table 3. List of scientific name, common name, code name, and accession numbers of DNA and amino acid sequences used in alignment and phylogenetic analyses. NA represents sequences that are unpublished and lack accession numbers. * represents sequences of putative partial bluegill G- protein fragments used in the current study (LmGi was characterized by Sandra Becerra).

Code used in tree	Scientific name	Common name	Gα Subtype	Accession # for nucleotide	Accession # for amino acid
RnG11	*Rattus norvegicus*	House rat	G11	AF239674	AAF81690
CgG11	*Cricetulus griseus*	Hamster	G11	DQ 202706	ABA77548
HsG11	*Homo sapiens*	Man	G11	AF493900	AAM12614
XlG11	*Xenopus laevis*	African clawed frog	G11	BC 042284	AAH42284
LmG11*	*Lepomis macrochirus*	Bluegill sunfish	G11	NA	NA
DrG11	*Danio rerio*	Zebrafish	G11	XM 686074	XP 691166
CgGq	*Cricetulus griseus*	Hamster	Gq	DQ202705	ABA77547
HsGq	*Homo sapiens*	Man	Gq	AF493896	AAM12610
MmGq	*Mus musculus*	Mouse	Gq	M 55412	AAA63306
RnGq	*Rattus norvegicus*	House rat	Gq	AF234260	AAF59930
LmGq*	*Lepomis macrochirus*	Bluegill sunfish	Gq	NA	NA
HsG14	*Homo sapiens*	Man	G14	BC027886	AAH27886
BtG14	*Bos taurus*	Cow	G14	NM 17323	NP 776748
MmG14	*Mus musculus*	Mouse	G14	NM 008137	NP 032163
MmG15	*Mus musculus*	Mouse	G15	M80632	AAA37713
MmGs	*Mus musculus*	Mouse	Gs	BC062654	AAH62654
RnGs	*Rattus norvegicus*	House rat	Gs	M12673	AAA41261
CgGs	*Cricetulus griseus*	Hamster	Gs	DQ202704	ABA77546
BtGs	*Bos taurus*	Cow	Gs	NM 181021	NP 851364
HsG13	*Homo sapiens*	Man	G13	NM 006572	NP 006563
HsG12	*Homo sapiens*	Man	G12	NM 007353	NP 031379
MmG12	*Mus musculus*	Mouse	G12	NM 010302	NP 034432
TrG12	*Takifugu rubripes*	Fugu	G12	L79907	AAL77634
HsGt1	*Homo sapiens*	Man	Gt1	BC095505	AAH95505
MmGt1	*Mus musculus*	Mouse	Gt1	NM 008140	NP 032166
DrGt1	*Danio rerio*	Zebrafish	Gt1	BC059464	AAH59464
XlGt1	*Xenopus laevis*	African clawed frog	Gt1	BC111509	AAI11510
MmGt2	*Mus musculus*	Mouse	Gt2	NM 008141	NP 032167
TrGi2	*Takifugu rubripes*	Fugu	Gi2	L79898	AAL77635
RnGi2	*Rattus norvegicus*	House rat	Gi2	NP_112297	NP_112297
MmGi1	*Mus musculus*	Mouse	Gi1	NM 010305	NP 034435
HsGi1	*Homo sapiens*	Man	Gi1	BC026326	AAH26326
GgGi1	*Gallus gallus*	Chick	Gi1	NM 205403	NP 990734

Table 3-Continued. List of scientific name, common name, code name, and accession numbers of DNA and amino acid sequences used in alignment and phylogenetic analyses. NA represents sequences that are unpublished and lack accession numbers. * represents sequences of putative partial bluegill G- protein fragments used in the current study (LmGi was characterized by Sandra Becerra).

Code used in tree	Scientific name	Common name	Gα Subtype	Accession # for nucleotide	Accession # for amino acid
MmGi3	*Mus musculus*	Mouse	Gi3	NM 010306	NP 034436
LmGi*	*Lepomis macrochirus*	Bluegill sunfish	Gi	NA	NA
MmGo	*Mus musculus*	Mouse	Go	NP_034438	NP_034438
RnGo	*Rattus norvegicus*	House Rat	Go	NM 017327	NP 059023
HsGo	*Homo sapiens*	Man	Go	NM 138736	NP 620073
MmGz	*Mus musculus*	Mouse	Gz	NM 010311	NP 034441
RnGz	*Rattus norvegicus*	House Rat	Gz	NM 013189	NP 037321
HsGz	*Homo sapiens*	Man	Gz	NM 002073	NP 002064
UmGpa1	*Ustilago maydis*	Fungus (smut)	Gpa1	UMU85775	AAC49724
McGpa1	*Mucor circinelloides*	Fungus (mold)	Gpa1	DQ286549	ABB85289

Immunolocalization of Gα$_{q/11}$ in fishes

The expression of Gα$_{11}$ and/or Gα$_q$ in bluegill and zebrafish eyes was studied to infer whether these G-proteins could play a role in light adaptive granular movements in the RPE. Towards that end, bluegill sunfish and zebrafish were killed by double pithing according to the IACUC protocols, and their eyes were excised and fixed in 4% paraformaldehyde in PBS (comprising 137 mM NaCl, 27mM KCl, 43 mM Na$_2$HPO$_4$ .7H2O and 14 mM KH$_2$PO$_4$, pH 8) overnight at 4°C. For zebrafish 7 days post fertilization (dpf),14 dpf and 21 dpf, the whole fish was fixed while for 28 dpf and adults, whole eyes were excised and fixed in 4% PFA in PBS. The tissue was washed three times in PBS and then transferred into vials containing 30% sucrose in PBS at 4°C overnight for cryoprotection. The samples were then washed and embedded in the embedding medium (Tissue-tek® O.C.T. compound, Sakura Finetek USA, Inc., product code # 4583) and the Tissue-tek containing samples were trimmed to 1mm cubes using razor blade. The tissue containing cubes were sectioned using a cryotome and the sections (10

micrometer thickness) were collected on coverslips coated with 0.5% gelatin. The

sections were dried at 4^oC overnight and were washed three times with phosphate

buffered saline containing 0.1% Tween-20 (PBST).

Sections were incubated in 20% non-fat powdered milk in PBS for 2 hours at

room temperature to block non-specific binding. The coverslips were washed three times

in PBST and were incubated in rabbit anti-mouse $G_{q/11}$ polyclonal antibody (Chemicon,

USA) (1:100) 2 hours at room temperature. The ability of the antibody to detect the $G_{q/11}$

protein in fish was tested first using the dot blot technique. Negative controls were

incubated in PBS. Coverslips were washed three times with PBST and were incubated

with Cy3-labeled anti-rabbit IgG (Invitrogen Inc, USA) for two hours at room

temperature (1:200). The coverslip was washed again in PBS and mounted on glass slides

in a medium consisting of 90% glycerol in water containing 1 mg/ml p-

phenylenediamine. Images were acquired with a confocal microscope using an Olympus

IX-70 fitted with a Bio-Rad MRC 1024 confocal scan-head via the Keller port and with

Olympus BH2-RFCA epifluorescence microscope fitted with Olympus C-35AD-4

camera.

CHAPTER III

RESULTS

Molecular characterization of a Gα$_{q/11}$ gene fragment from bluegill brain cDNA

In order to molecularly characterize members of the Gα$_{q/11}$ gene family, primers were first designed to amplify Gα$_{11}$. Using the primer set G11F1 and G11R1 shown in Table 2, an approximately 500 bp DNA fragment was amplified from bluegill brain cDNA. The sequenced products from both primers formed a single, bidirectional, contiguous sequence, and the second primer set G11F2 and G11R2 (see Table 2) was designed based on this sequence to obtain the non-overlapping 3' region. A third primer set, G11F3 and G11R3 (see Table 2), was also designed to obtain the non-overlapping 5' region. The fragments obtained using the three primer sets and their positions relative to the contiguous sequence are shown in Figure 3. All three sequence fragments when joined generated an ~ 893 bp contiguous sequence. Using BLAST, this sequence was compared with G-protein sequences from other vertebrates, and it showed highest identity (up to 85 %) with Gα$_{11}$ sequences. The Gα$_{11}$-like fragment retrieved from bluegill was compared with Gα$_{11}$ in mammals (see Figure 4) and the results showed that out of the seven exons, the retrieved bluegill gene fragment was missing only exon 1 and a portion of exon 2, exons 3-7 being fully sequenced. The 893 bp long sequence was translated in

six reading frames, and a single open reading frame encoding 296 amino acids was encoded in frame 2 (Figure 3).

The deduced amino acid sequence when compared with other vertebrate G-protein sequences also showed highest homology (up to 99%) with $G\alpha_{11}$ sequences. Botthe nucleotide and the deduced amino acid sequences of the bluegill $G\alpha_{11}$-like fragment are shown in Figure 5. An alignment of the deduced amino acid sequence of the bluegill $G\alpha_{11}$-like fragment with known vertebrate sequences is shown in Figure 6. The percentage identity and similarity between this sequence and vertebrate $G\alpha_{11}$ sequences is shown in Table 4. The highest percent identity and similarity was observed with a zebrafish (97% and 99%, respectively) $G\alpha_{11}$-like sequence. Since $G\alpha_{11}$ is known to be highly similar to $G\alpha_q$ in mammals, the percent similarity of the putative bluegill $G\alpha_{11}$ fragment with $G\alpha_q$ sequences from other vertebrates was also evaluated (see Table 5). In fact, based on the comparison of fish and mammalian $G\alpha_{11}$ and $G\alpha_q$ "signature" residues, I find that of the 21 residues inferred for bluegill $G\alpha_{11}$-like fragment (highlighted in green in Figure 6), 9 residues are $G\alpha_q$-like.
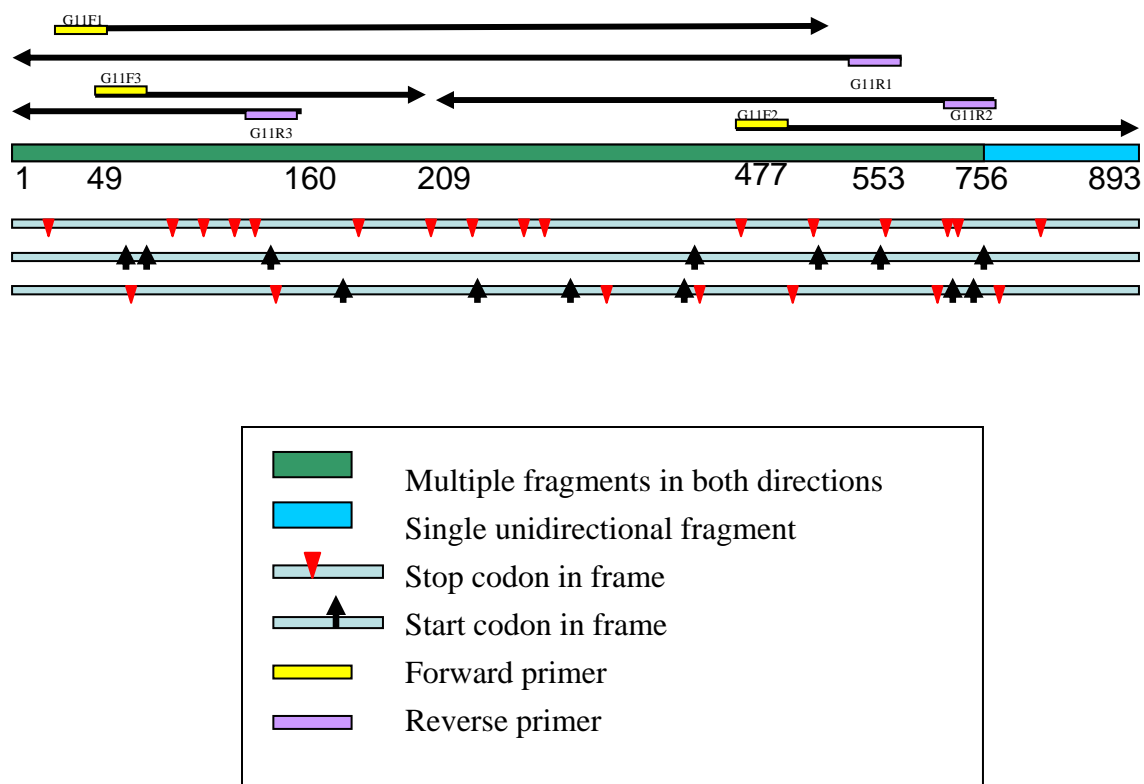
Figure 3. Position of PCR fragments for the Gα₁₁-like fragment obtained using primers listed in Table 2. The position of primers relative to the sequence and their direction are also denoted in the figure. The horizontal black lines represent the gene fragments generated from the primers given in Table 2. The names of the primers are provided above or below the primer.
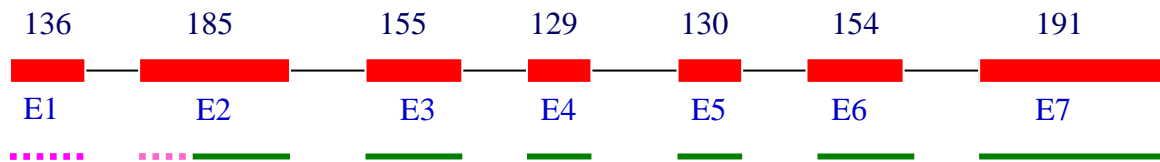
Figure 4. A comparison of the gene structure of mammalian Gα₁₁ with the Gα₁₁-like gene fragment retrieved from bluegill cDNA. The exons E1-E7 are shown as red boxes and are interrupted by 6 introns (represented as black lines). The numbers above the red boxes indicate the expected length of each exon in mammalian Gα₁₁ sequences. The lines in green represent the length of the exons retrieved in the current study from bluegill cDNA (~893 bp) and the dotted pink lines represent the part of the gene still uncharacterized from bluegill (~193 bp). This diagram is not to scale and is based on the output from Ensembl comparison of the bluegill Gα₁₁-like gene fragment with mammalian Gα₁₁ sequences.

```
tggagccgggtactcagacgaggataagagaggcttcatccgccttgtttaccaaaacatc
  G   A   G   Y   S   D   E   D   K   R   G   F   I   R   L   V   Y   Q   N   I
ttcacctccatgcagtccatgatccgtgccactgagacccttaagatcccctacaaattt
  F   T   S   M   Q   S   M   I   R   A   T   E   T   L   K   I   P   Y   K   F
gaacagaatcggtctaacgccatgcttgtgaaagaggtggacatcgagaagatcaatggg
  E   Q   N   R   S   N   A   M   L   V   K   E   V   D   I   E   K   I   N   G
tttgaccatccctacattgtggctatcaaaagcctgtgggctgacccagggatccaagag
  F   D   H   P   Y   I   V   A   I   K   S   L   W   A   D   P   G   I   Q   E
gcctatgaccgccgcagagagtaccagctctctgactccactaaatattatctttccgat
  A   Y   D   R   R   R   E   Y   Q   L   S   D   S   T   K   Y   Y   L   S   D
ctggatcgtattgcagattccaactatcttcccactcagcaggatgtgctcagggtgcgc
  L   D   R   I   A   D   S   N   Y   L   P   T   Q   Q   D   V   L   R   V   R
atccccactacaggaatcatagagtatccattcgacttgcaaagcatcattttcaggatg
  I   P   T   T   G   I   I   E   Y   P   F   D   L   Q   S   I   I   F   R   M
gtggatgtaggggggtcagaggtcagagaggaggaagtggattcactgctttgagaacgtc
  V   D   V   G   G   Q   R   S   E   R   R   K   W   I   H   C   F   E   N   V
acctccattatgtttctggtggccctgagtgagtacgaccaggtcctggtggaatcagac
  T   S   I   M   F   L   V   A   L   S   E   Y   D   Q   V   L   V   E   S   D
aacgagaaccgcatggaggagagtaaagctctgttcaggactatcattacgtacccctgg
  N   E   N   R   M   E   E   S   K   A   L   F   R   T   I   I   T   Y   P   W
tttcaaaactcctccgttatcctcttcctcaacaagaaggacctgctagaggagaagatc
  F   Q   N   S   S   V   I   L   F   L   N   K   K   D   L   L   E   E   K   I
gcctactcacacttggtggactatttccctgagtttgatggtccacagagagatgcacag
  A   Y   S   H   L   V   D   Y   F   P   E   F   D   G   P   Q   R   D   A   Q
gcagcgcgggagttcatcctcaagatgtttgtggacttaaacccagacagcgacaagatc
  A   A   R   E   F   I   L   K   M   F   V   D   L   N   P   D   S   D   K   I
atctactctcacttcacttgtgccacggacactgagaacatccgctttgtgtttgcagct
  I   Y   S   H   F   T   C   A   T   D   T   E   N   I   R   F   V   F   A   A
gtcaaagacaccatcctacagctcaatctcaaagagtacaatctggtgtga
  V   K   D   T   I   L   Q   L   N   L   K   E   Y   N   L   V   -
```

Figure 5. **Nucleotide sequence of bluegill Gα₁₁-like fragment and the deduced amino acid residues**. The nucleotide sequence is 893 bp long (in black), and there are 296 deduced amino acid residues (in blue).
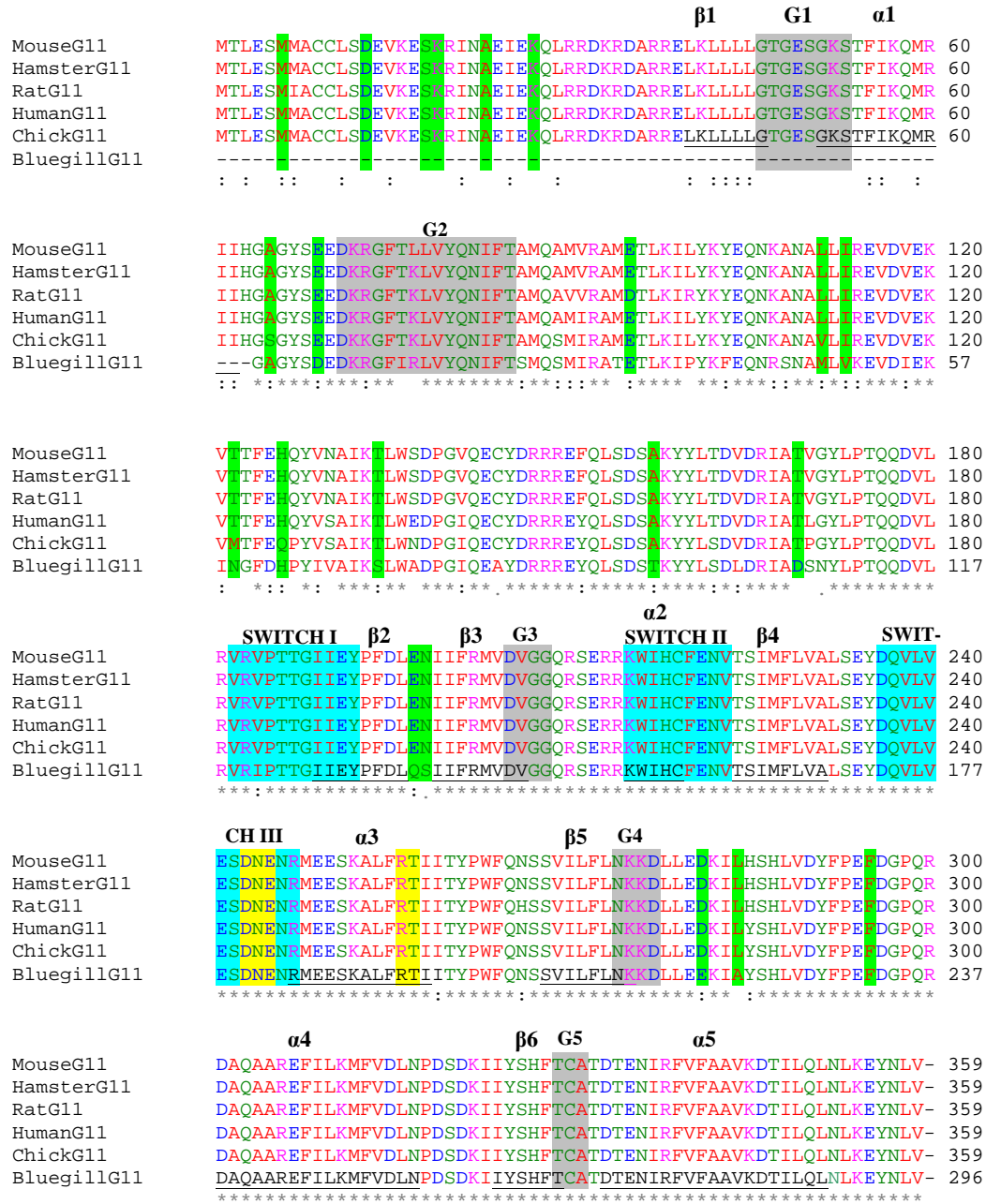
Figure 6. Alignment of deduced amino acid sequences of bluegill Gα₁₁-like fragment with published vertebrate Gα₁₁ sequences using CLUSTAL W. The G1-G5 boxes (GTP-binding domains) are highlighted in grey and switches I-III are highlighted in blue. The amino acid residues highlighted in green represents the residues that differ between Gα₁₁ and Gαq, while those highlighted in yellow represent the residues critical for binding with phospholipase C-β. The residues constituting the α1-α5 helices and β1-β5 sheets identified from the crystalline structure of Gαt are underlined in black. The stars at the bottom of the alignment indicate homology, and the double dots represent amino acids that are conserved but not identical among all organisms. The accession numbers of the sequences used in the alignment are provided in Table 4.

Table 4. **Table showing percent identity and percent similarity of bluegill Gα₁₁-like fragment and Gα_q fragment with Gα₁₁ fragments of other vertebrates at the amino acid level using BL2SEQ program.** The numbers in black represent percent identity and numbers in parentheses represent percent similarity. * indicates a predicted sequence.

Sequence	Scientific name	Accession number	% identity to bluegill Gα₁₁	% identity to bluegill Gα_q
Human Gα₁₁	*Homo sapiens*	AAM12614	88 (95)	86 (93)
Mouse Gα₁₁	*Mus musculus*	NP_034431	85 (95)	84 (93)
Rat Gα₁₁	*Rattus norvegicus*	AAF81690	85 (95)	84 (93)
Hamster Gα₁₁	*Cricetulus griseus*	ABA77548	86 (95)	85 (93)
Chick Gα₁₁	*Gallus gallus*	NP_989565	88 (95)	84 (94)
Zebrafish Gα₁₁*	*Danio rerio*	AHH85433	97 (99)	85 (94)

Molecular characterization of Gα_q gene fragment from bluegill brain cDNA

The primers for Gα_q listed in Table 2 were used to amplify ~741 bp DNA fragment from bluegill brain cDNA. The products from the primer set GqF and GqR generated a single, bidirectional, contiguous sequence (~692 bp). The sequence fragments obtained using the primer set and their positions relative to the contiguous sequence are shown in Figure 7. This sequence was compared with G-protein sequences from other vertebrates, and it showed highest homology with Gα_q sequences (up to 85%). The sequence was translated in six reading frames and a single open reading frame encoding 230 amino acids was encoded in frame 3 (see Figure 7). The deduced amino acid sequence when compared with other vertebrate G-protein sequences also showed highest homology with Gα_q sequences (up to 98%). Both the nucleotide and the deduced amino acid sequences of the putative bluegill Gα_q are shown in Figure 8. A comparison of the gene structure of Gα_q (in mammals) and the Gα_q fragment retrieved from bluegill cDNA is given in Figure 9, and the results showed that out of the seven exons in the expected mammalian sequences, the retrieved bluegill Gα_q gene fragment was missing

exon 6 and 7 and the first forty-four residues of exon 1, the rest being fully sequenced. An alignment of the deduced amino acid sequence of bluegill $G\alpha_q$ with other vertebrate $G\alpha_q$ sequences is shown in Figure 10. Upon comparing bluegill and mammalian $G\alpha_{11}$ and $G\alpha_q$ "signature" residues, it was observed that of the 21 residues inferred for bluegill $G\alpha_q$ (highlighted in green in Figure 10), only two residues appear to be $G\alpha_{11}$-like. The percentage identity and similarity between this sequence and other vertebrate $G\alpha_q$ sequences is shown in Table 5.
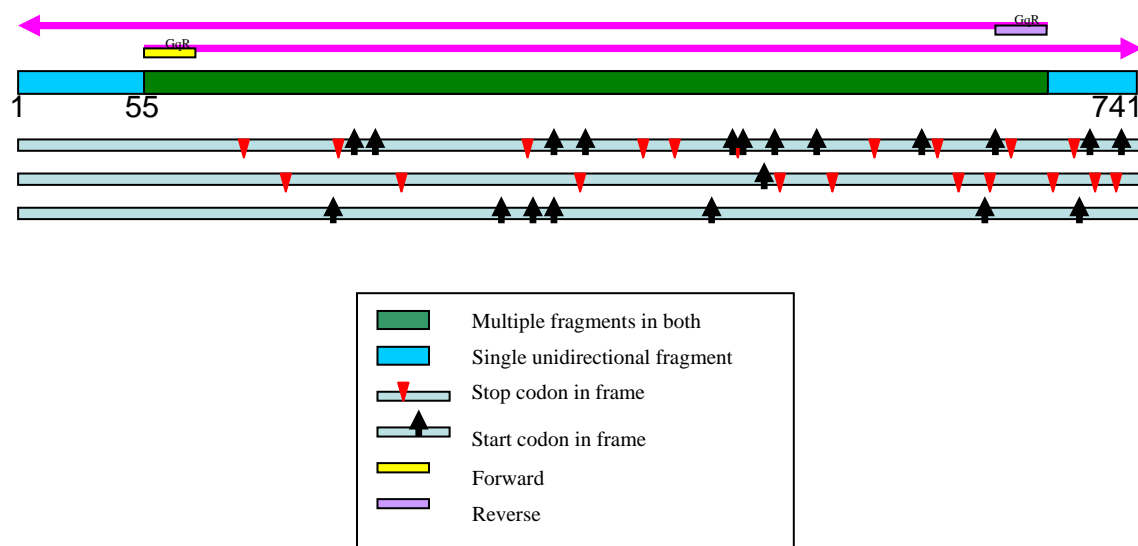
Figure 7. Position of PCR fragments for bluegill Gα_q obtained using primers listed in Table 2. The position of primers relative to the sequence and their direction are also denoted in the figure. The horizontal black arrows represent the gene fragments generated from the primers given in Table 2, and the primer names are provided above the respective primers.

```
gcaaggaagccaggcggatcaacgacgagatcgaaaggcagctccgccgggacaagagggac
   K   E   A   R   R   I   N   D   E   I   E   R   Q   L   R   R   D   K   R   D
gcacgccgggaactgaaactgttgcttctcggcaccggggaaagtggtaagagcacattc
  A   R   R   E   L   K   L   L   L   L   G   T   G   E   S   G   K   S   T   F
atcaaacagatgaggattatccatggcaccggctactccgatgaggacaaaagaggtttc
  I   K   Q   M   R   I   I   H   G   T   G   Y   S   D   E   D   K   R   G   F
accaaactggtctatcagaacatcttcacagccatgcaggccatgatccgagccatggag
  T   K   L   V   Y   Q   N   I   F   T   A   M   Q   A   M   I   R   A   M   E
acgctcaagatcccctacaaatatgagcacaacaagggcaatgccaacattgtgagagag
  T   L   K   I   P   Y   K   Y   E   H   N   K   G   N   A   N   I   V   R   E
gtggacgtagaaaaggtctccatgttcgagaatccttatgtagatgcaatcaagagctta
  V   D   V   E   K   V   S   M   F   E   N   P   Y   V   D   A   I   K   S   L
tggaatgacccaggaatccaggagtgctatgatcgaaggagggaataccaactctcagac
  W   N   D   P   G   I   Q   E   C   Y   D   R   R   R   E   Y   Q   L   S   D
tctaccaaatattacctgaacgcgttggatcggatcgccgagccagcctaccttcccact
  S   T   K   Y   Y   L   N   A   L   D   R   I   A   E   P   A   Y   L   P   T
cagcaggatgtgttgagggttagagtcccccaccacgggcatcatgcaatacccgtttgac
  Q   Q   D   V   L   R   V   R   V   P   T   T   G   I   M   Q   Y   P   F   D
ctgcagagtgtcatattcaggatggtggatgtaggaggtcagaggtcggagaggaggaag
  L   Q   S   V   I   F   R   M   V   D   V   G   G   Q   R   S   E   R   R   K
tggatccactgttttgagaacgtcacatccattatgttcctggtagcgctcagcgagtat
  W   I   H   C   F   E   N   V   T   S   I   M   F   L   V   A   L   S   E   Y
gaccaagttttggtggaatcagacaatgaga
  D   Q   V   L   V   E   S   D   N   E
```

Figure 8. Nucleotide sequence of the bluegill Gα$_q$ fragment and the deduced amino acid residues. The nucleotide sequence is 692 bp long, and there are 230 deduced amino acid residues.
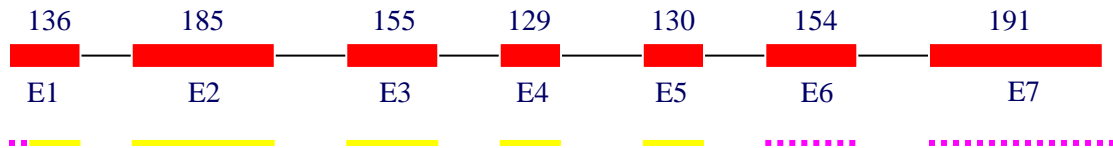
Figure 9. A comparison of the gene structure of mammalian Gα_q with the Gα_q gene fragment retrieved from bluegill cDNA. The exons E1-E7 are shown as red boxes and are interrupted by 6 introns (represented as black lines). The numbers above the red boxes indicate the length of each exon (in base pairs) in mammalian Gα_q sequences. The lines in yellow represent the length of the exons retrieved in the current study from bluegill cDNA (~692 bp) and the dotted pink lines represent the part of the gene still uncharacterized from bluegill (~44 bp on the 5' end of the sequence and ~344 bp on the 3' end of the sequence). This diagram is not to scale and is based on the output from Ensembl comparison of the bluegill Gα_{11}-like gene fragment with mammalian Gα_{11} sequences.

```
                         β1        G1          α1
MouseGq    MTLESIMACCLSEEAKEARRINDEIEQLRRDKRDARRELKLLLLGTGESGKSTFIKQMR 60
RatGq      MTLESIMACCLSEEAKEARRINDEIEQLRRDKRDARRELKLLLLGTGESGKSTFIKQMR 60
HamsterGq  MTLESIMACCLSEEAKEARRINDEIEQLRRDKRDARRELKLLLLGTGESGKSTFIKQMR 60
HumanGq    MTLESIMACCLSEEAKEARRINDEIEQLRRDKRDARRELKLLLLGTGESGKSTFIKQMR 60
ChickGq    MTLESIMACCLSEEAKEARRINDEIEQLRRDKRDARRELKLLLLGTGESGKSTFIKQMR 60
BluegillGq -----L--------KEARRINDEIEQLRRDKRDARRELKLLLLGTGESGKSTFIKQMR 45
                     **::***  ***:*********************************

                         G2
MouseGq    IIHGSGYSDEDKRGFTKLVYQNIFTAMQAMIRAMDTLKIPYKYEHNKAHAQLVREVDVEK 120
RatGq      IIHGSGYSDEDKRGFTKLVYQNIFTAMQAMVRAMDTLKIPYKYEHNKAHAQLVREVDVEK 120
HamsterGq  IIHGSGYSDEDKRGFTKLVYQNIFTAMQAMIRAMDTLKIPYKYEHNKAHAQLVREVDVEK 120
HumanGq    IIHGSGYSDEDKRGFTKLVYQNIFTAMQAMIRAMDTLKIPYKYEHNKAHAQLVREVDVEK 120
ChickGq    IIHGSGYSDEDKRGFTKLVYQNIFTAMQAMIRAMDTLKIPYKYEHNKAHAQLVREVDVEK 120
BluegillGq IIHGTGYSDEDKRGFTKLVYQNIFTAMQAMIRAMETLKIPYKYEHNKGNANIVREVDVEK 105
           ****:***:*********************:***:**** ****:** .:* ::*******

MouseGq    VSAFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYYLNDLDRVADPSYLPTQQDVL 180
RatGq      VSAFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYYLNDLDRVADPSYLPTQQDVL 180
HamsterGq  VSAFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYYLNDLDRVADPSYLPTQQDVL 180
HumanGq    VSAFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYYLNDLDRVADPAYLPTQQDVL 180
ChickGq    VSTFENPYVDAIRSLWNDPGIQECYDRRREYQLSDSTKYYLNDLDRIADSTYLPTQQDVL 180
BluegillGq VSMFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYYLNALDRIAEPAYLPTQQDVL 165
           *: ***********************************:****:*::*********

                                                 α2
                 β2         β3  G3       SWITCH II    β4       SWIT-
MouseGq    RVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV 240
RatGq      RVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV 240
HamsterGq  RVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV 240
HumanGq    RVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV 240
ChickGq    RVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV 240
BluegillGq RVRVPTTGIMQYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV 225
           *********::*****.*************************************

           CH III     α3              β5  G4
MouseGq    ESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR 300
RatGq      ESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR 300
HamsterGq  ESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR 300
HumanGq    ESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR 300
ChickGq    ESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR 300
BluegillGq ESDNE--------------------------------------------------- 230
           *****  :      ::   ::    :      :::::     ::      ::      ::

                α4            β6 G5            α5
MouseGq    DAQAAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVAAVKDTILQLNLKEYNLV 359
RatGq      DAQAAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVAAVKDTILQLNLKEYNLV 359
HamsterGq  DAQAAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVAAVKDTILQLNLKEYNLV 359
HumanGq    DAQAAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVAAVKDTILQLNLKEYNLV 359
ChickGq    DAQAAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVAAVKDTILQLNLKEYNLV 359
BluegillGq -----------------------------------------------------------
                ::: ::: :      ::    :       : ::: :   :: : :      ::
```
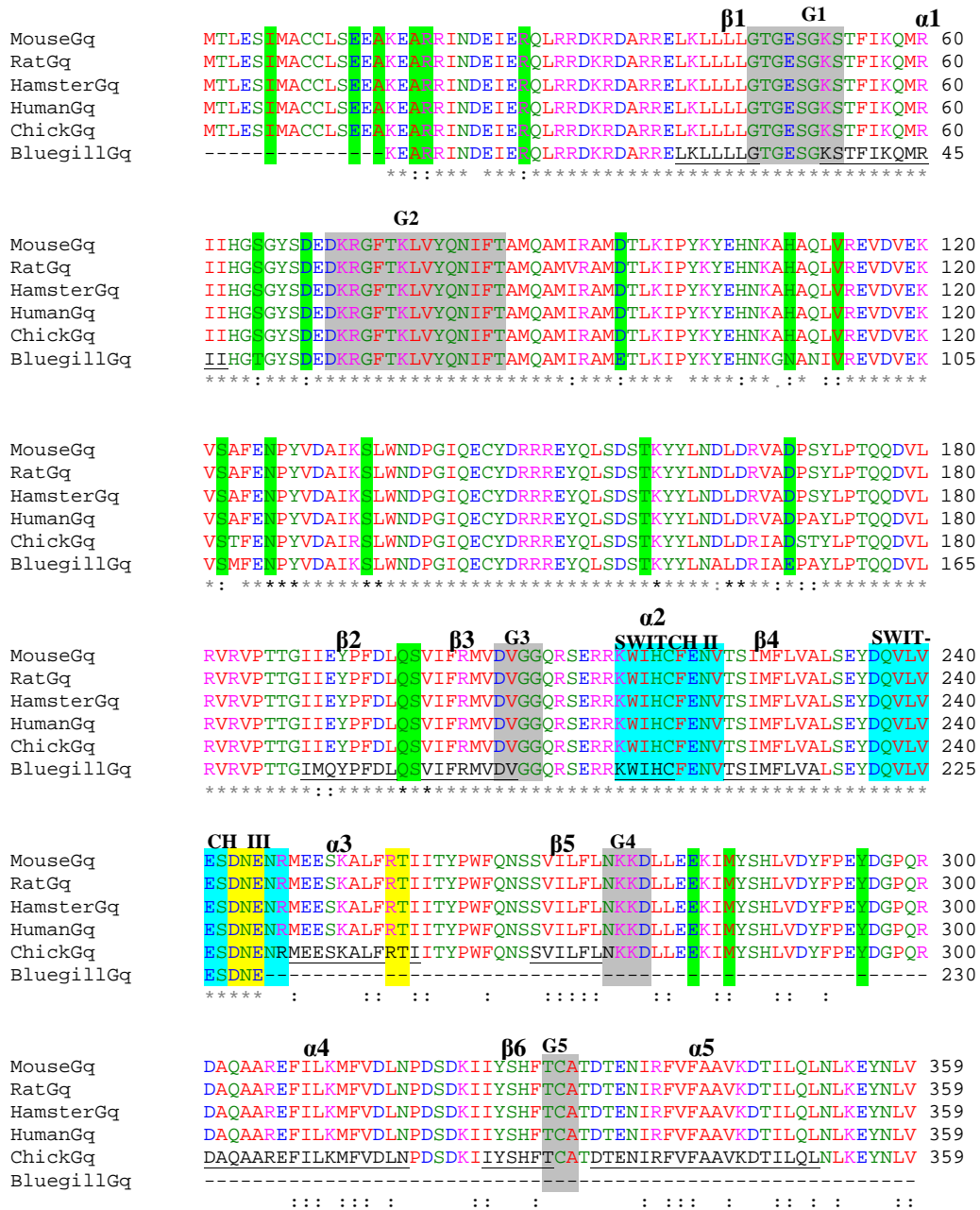
Figure 10. Alignment of deduced amino acid sequence of bluegill Gα$_q$ fragment with published vertebrate Gα$_q$ sequences using CLUSTAL W. The G1-G5 boxes (GTP binding domains) are highlighted in grey and switches I-III highlighted in blue. The amino acid residues highlighted in green represents the residues that are different between Gα$_{11}$ and Gα$_q$ while those highlighted in yellow represents the residues that are critical for binding with phospholipase C-β. The residues constituting the α1-α5 helices and β1-β5 sheets of the crystalline structure are underlined in black. The stars at the bottom of the alignment represent homology, and the double dots represent amino acids conserved but not identical among organisms. The accession numbers of the sequences used in the alignment are provided in Table 5.

Table 5. **Table showing percent identity and percent similarity of bluegill putative Gα$_q$ and Gα$_{11}$ fragments with Gα$_q$ fragments of other vertebrates at the amino acid level using BL2SEQ program.** The numbers represent percent identity, and numbers in parentheses represent percent similarity.

Sequence	Scientific name	Accession number	% identity to bluegill Gα$_q$	% identity to bluegill Gα$_{11}$-like fragment
Human Gα$_q$	*Homo sapiens*	AAM12610	94 (98)	89 (96)
Mouse Gα$_q$	*Mus musculus*	AAA63306	94 (98)	89 (96)
Rat Gα$_q$	*Rattus norvegicus*	AA59930	93 (98)	88 (96)
Chick Gα$_q$	*Gallus gallus*	NP_001026598	93 (97)	89 (96)

Phylogenetic analyses

The phylogenetic signal for the dataset was estimated to be -0.576, indicating a strong signal. In all tree topologies obtained using protein and nucleotide alignments employing different phylogenetic methods, mostly similar grouping of G-protein subtypes were observed although the resolution and bootstrap statistical support for some taxa varied among the methods employed. In all tree topologies, the Gα-subtypes grouped to form a single in-group. Within the in-group, they formed two monophyletic units. One unit consisted of Gα$_{11}$, Gα$_q$, Gα$_{14}$, Gα$_{15}$, Gα$_{12}$, Gα$_{13}$ and Gα$_s$, and the other group consisted of Gα$_{t1}$, Gα$_{t2}$, Gα$_{i1}$, Gα$_{i2}$, Gα$_{i3}$, Gα$_z$ and Gα$_o$ subtypes. In all trees obtained using parsimony, neighbor joining, maximum likelihood and Bayesian analysis of the nucleotide alignment (Figures 9-12, respectively), the functional differences among and within Gα subtypes were clearly reflected. In general, all four members of the G$_{q/11}$ family that activate phospholipase C were grouped together and were paraphyletic with Gα$_{12/13}$ and Gα$_s$ families, both of which were paraphyletic to one another. The Gα$_i$ group that inhibits adenylyl cyclase were grouped together in all tree topologies. The bluegill

$G\alpha_{q/11}$ sequence retrieved using primers designed based on mammalian $G\alpha_{11}$ sequences formed a sister taxon with zebrafish $G\alpha_{11}$ sequence and was paraphyletic with mammalian and amphibian $G\alpha_{11}$ sequences. Though the mammalian $G\alpha_{11}$ sequences grouped to form a single monophyletic unit, the lower vertebrate $G\alpha_{11}$ sequences were grouped with the $G\alpha_q$ sequences.

The putative bluegill $G\alpha_q$ formed a sister group with all mammalian orthologs of $G\alpha_q$, and all taxa grouped to form a single monophyletic unit. High bootstrap support (100%) was observed in the grouping of the bluegill $G\alpha_{11}$-like fragment with the zebrafish $G\alpha_{11}$-like gene; the support for bluegill $G\alpha_q$ was slightly lower. The parsimony tree obtained using the protein alignment (Figure 15) showed mostly similar grouping to that of the topology obtained using nucleotide alignment. One major difference observed in the protein-based tree topology was the unresolved $G\alpha_s$ group. In this topology, three distinct monophyletic in-groups (instead of two as in other trees) were formed with $G\alpha_q$ and $G\alpha_{12/13}$ families grouped together, the $G\alpha_s$ family and the $G\alpha_i$ family, all of which form a polychotomous group. High bootstrap support (>70%) was observed for both bluegill sequences in the protein alignment. The putative bluegill $G\alpha_i$ (identified and characterized by Sandra Becerra) was grouped with other $G\alpha_i$ members and was closer to $G\alpha_{i1}$ and $G\alpha_{i3}$ rather than $G\alpha_{i2}$. High bootstrap support (>90%) was observed for this grouping in both the nucleotide-based and protein-based tree topologies.
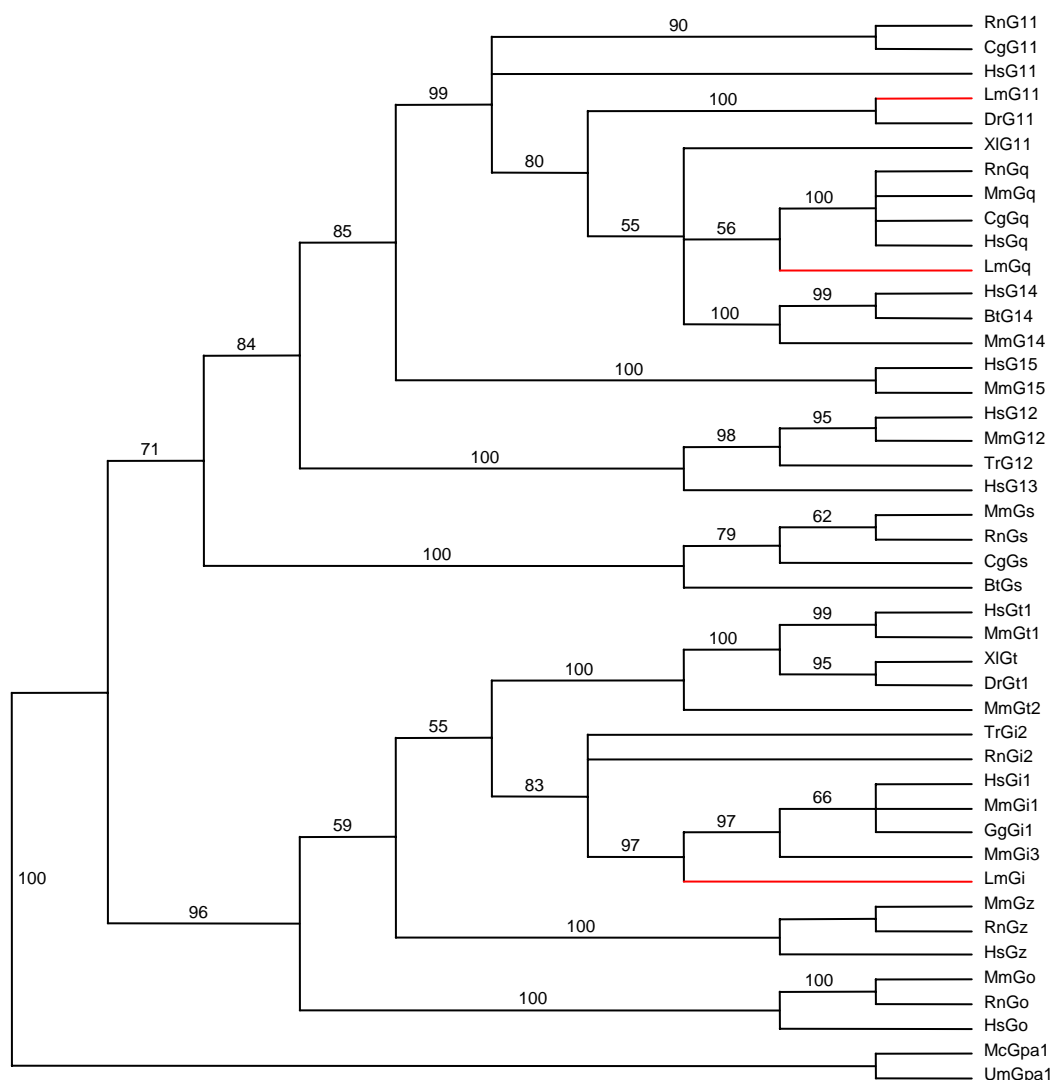
Figure 11. Consensus parsimony tree for nucleotide alignment obtained by heuristic search of the dataset. The numbers indicate bootstrap statistical support values obtained using five thousand random replications of the dataset. Red lines indicate unpublished putative partial G-protein sequences from bluegill sunfish. The codes for various taxa and their scientific names are given in Table 3.
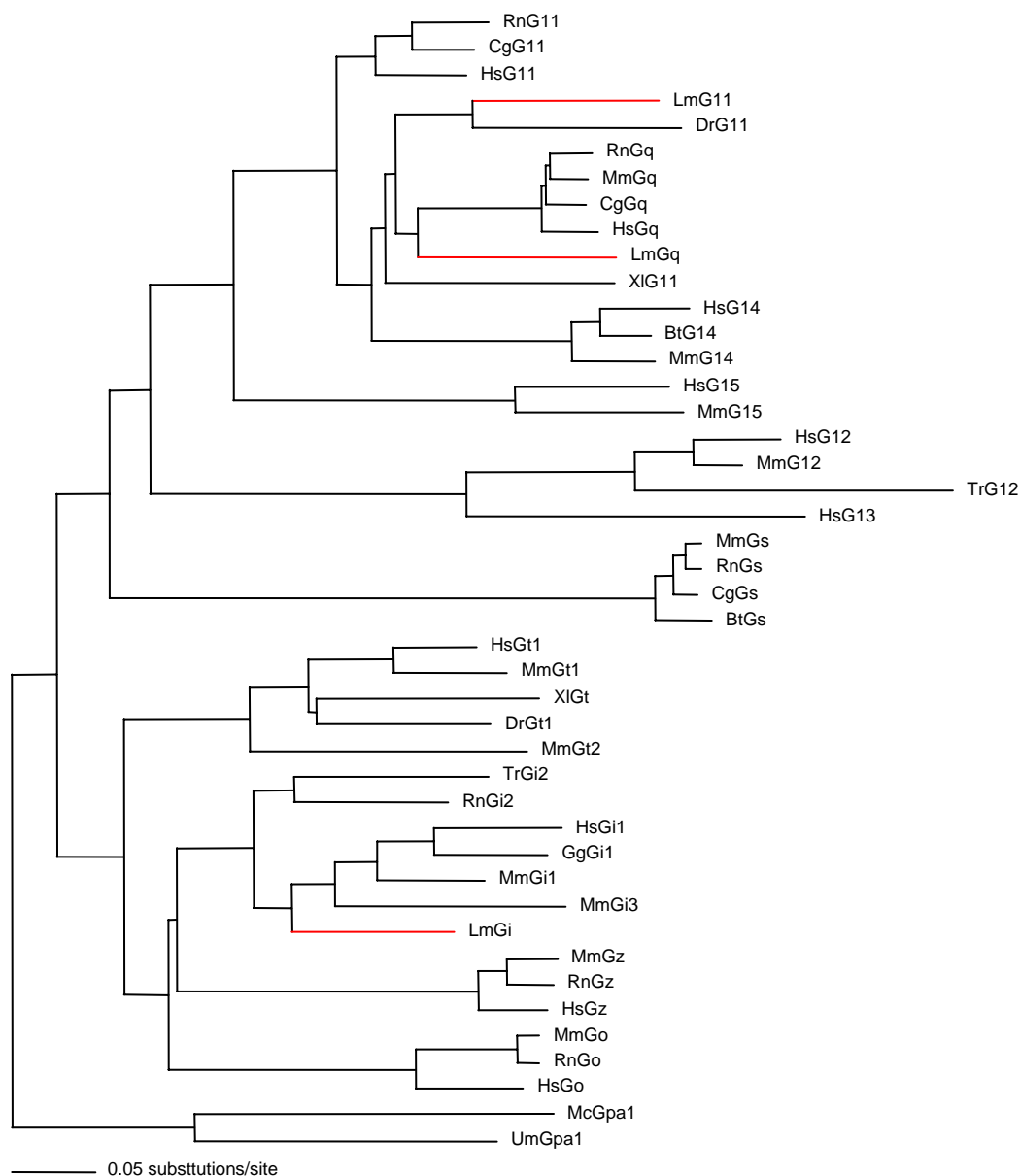
Figure 12. Neighbor joining tree for DNA alignment with Tamura-Nei model as distance correction obtained by heuristic search with five thousand random stepwise replications. The red lines indicate unpublished putative partial G-protein sequences from bluegill sunfish. The codes for taxa and their scientific names are given in Table 3.

Figure 13. The consensus maximum likelihood tree topology using GTR+I+G model of evolution. The numbers represent bootstrap statistical support values obtained using fifty random replications of the dataset using heuristic search. The red lines indicate unpublished putative partial G-protein sequences from bluegill sunfish. The codes for taxa and their scientific names are given in Table 3.
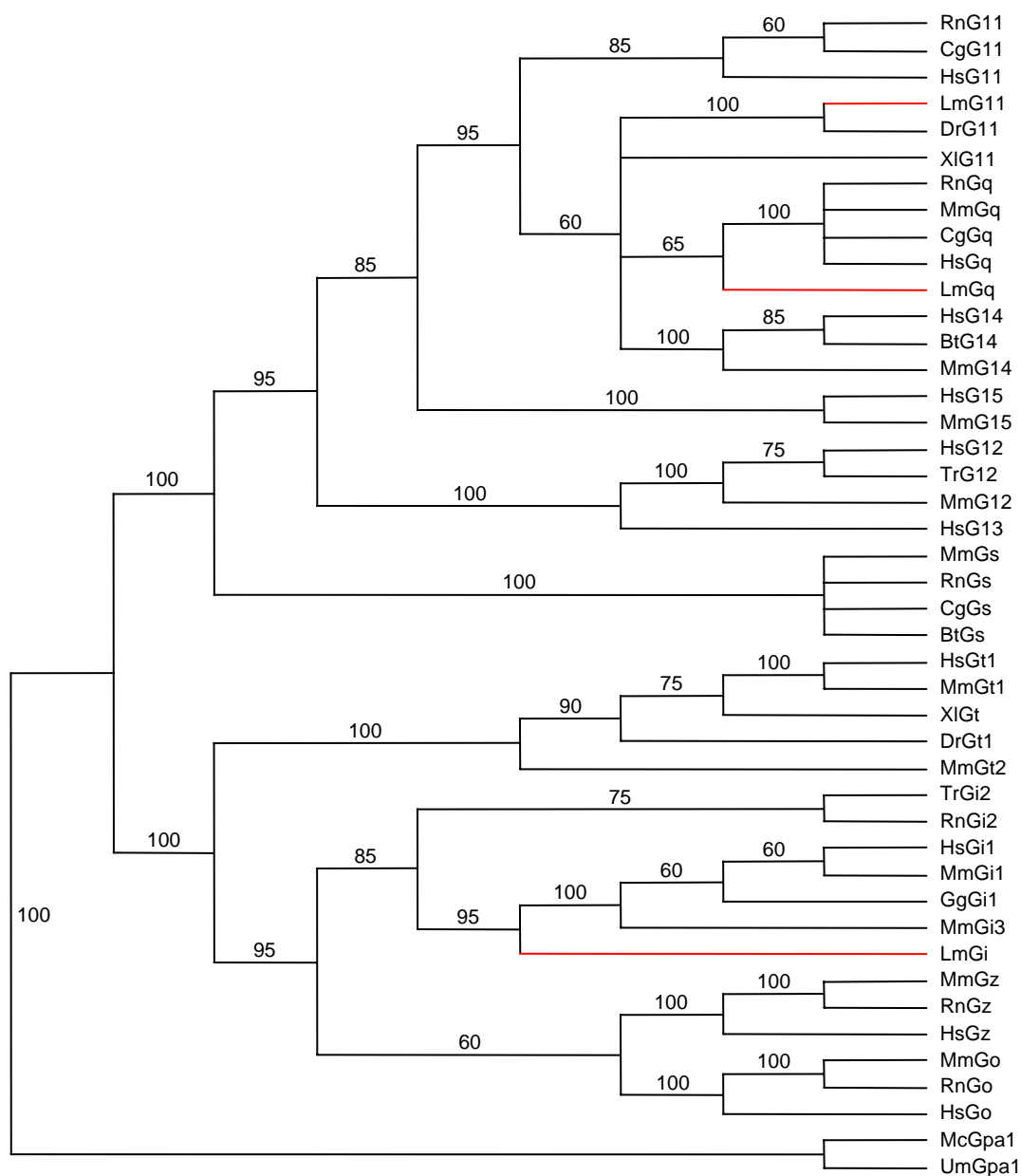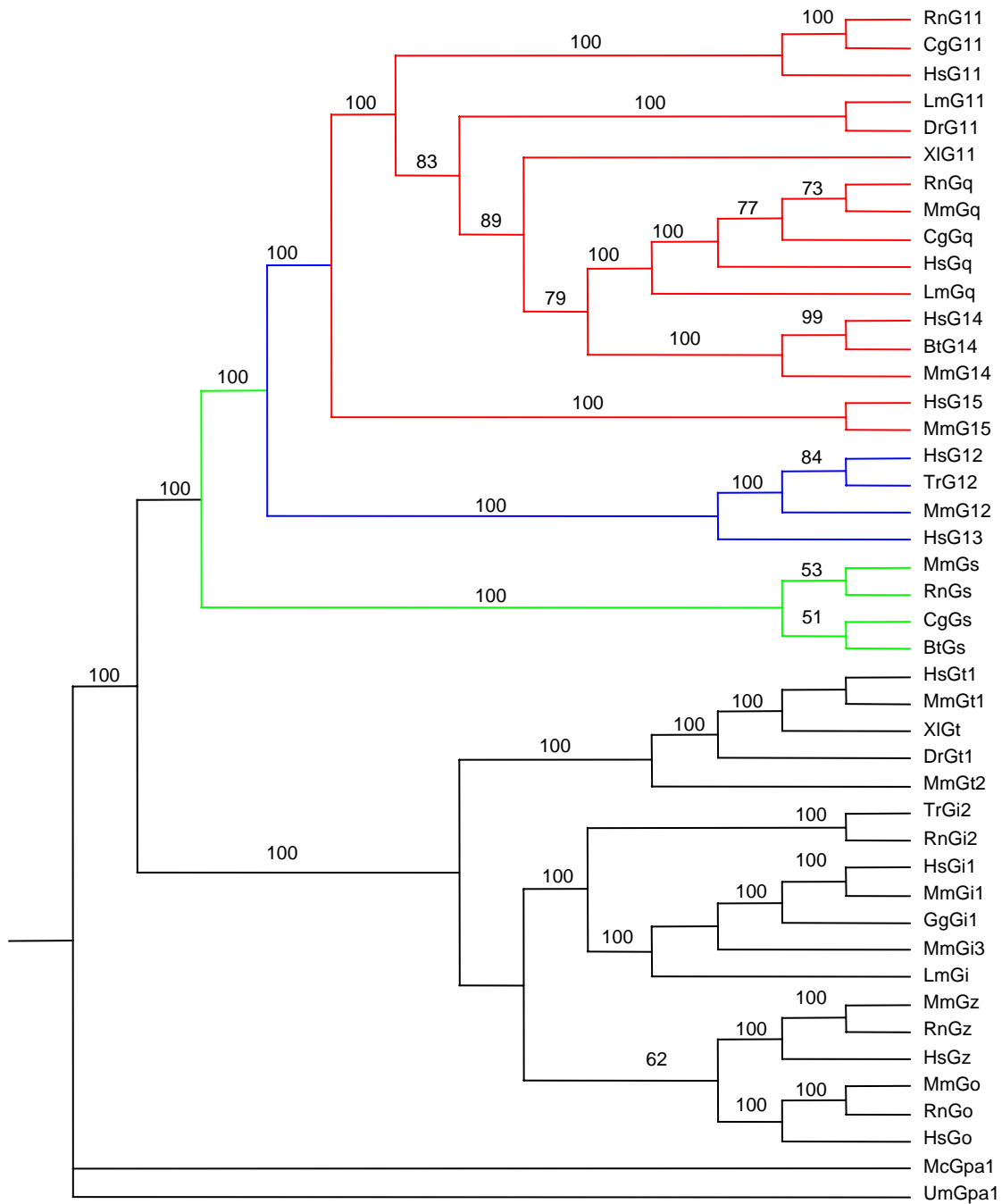
Figure 14. Tree showing Bayesian analysis of posterior probability using the GTR+I+G model of evolution. The numbers represent the posterior probability values and Gα sequences are classified based on their function into four families and are color coded. Red lines represent Gα$_q$ family, blue represents Gα$_{12/13}$, green represents Gα$_s$ and Gα$_i$ is denoted by black lines. The codes for taxa and their scientific names are given in Table 3.

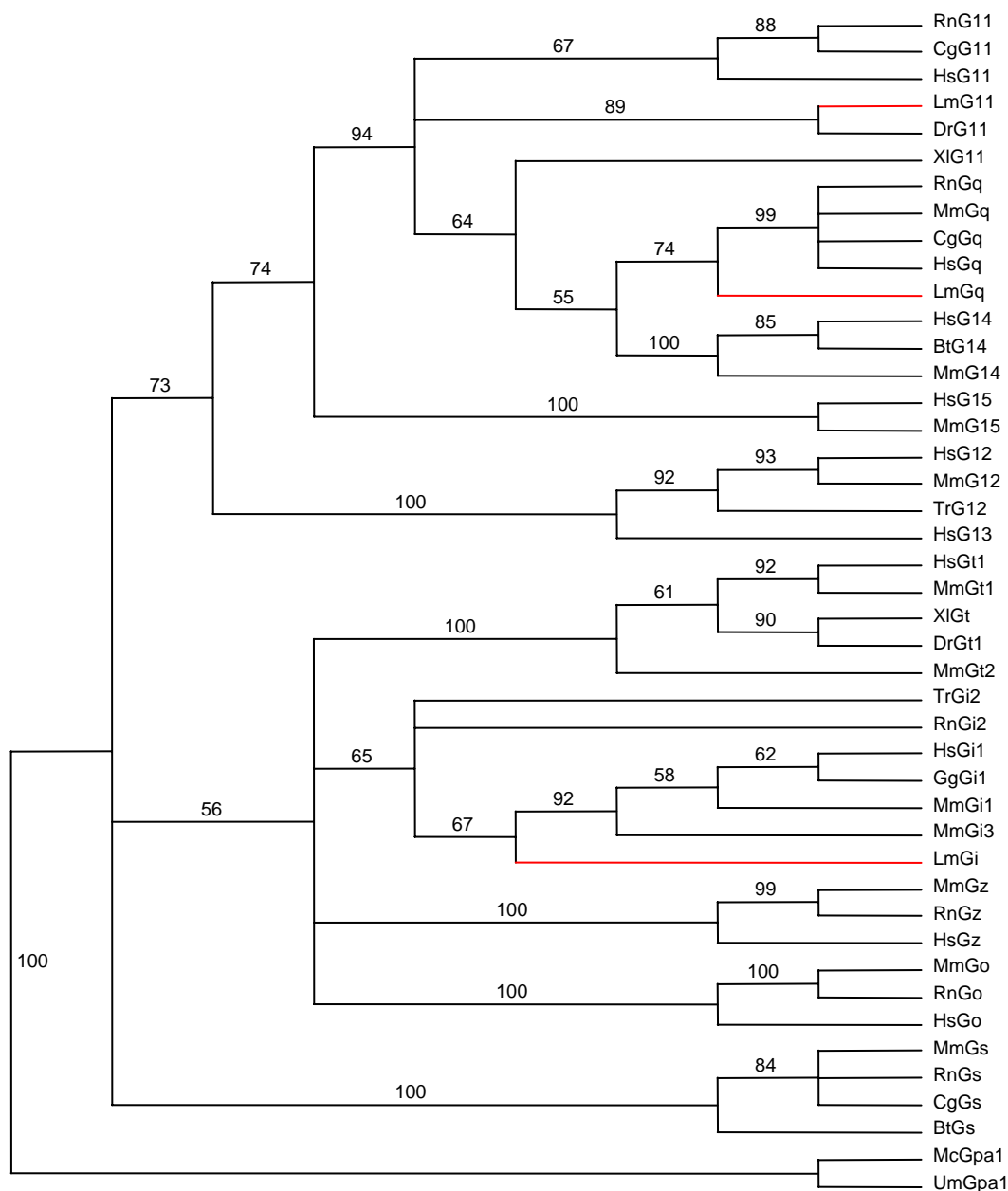**Figure 15. Consensus parsimony tree for protein alignment obtained using heuristic search of the dataset.** The numbers indicate bootstrap statistical support values obtained using five thousand random replications of the dataset. Red lines indicate unpublished putative partial G-protein sequences from bluegill sunfish. The codes for various taxa and their scientific names are given in Table 3.

RT-PCR analysis

To assess if $G\alpha_q$ and $G\alpha_{11}$-like mRNA are expressed in fish, RT-PCR was

performed on total RNA extracted from bluegill brain, heart, retina and RPE and from

zebrafish brain, heart and whole eyes using the primers shown in Table 2. Figure 16

shows that while $G\alpha_{11}$-like gene ($G\alpha_{10}$) is expressed in fish brain, $G\alpha_q$ is expressed in all

tissues tested. β-actin, expressed ubiquitously in all tissues, served as positive controls for

the cDNAs. When the $G\alpha_q$ primers and primers designed to amplify bluegill $G\alpha_{11}$ were

used to perform RT-PCR on total RNA isolated from zebrafish, only $G\alpha_q$ and β-actin

primers were successful in amplifying the respective genes. $G\alpha_{10}$ primers designed for

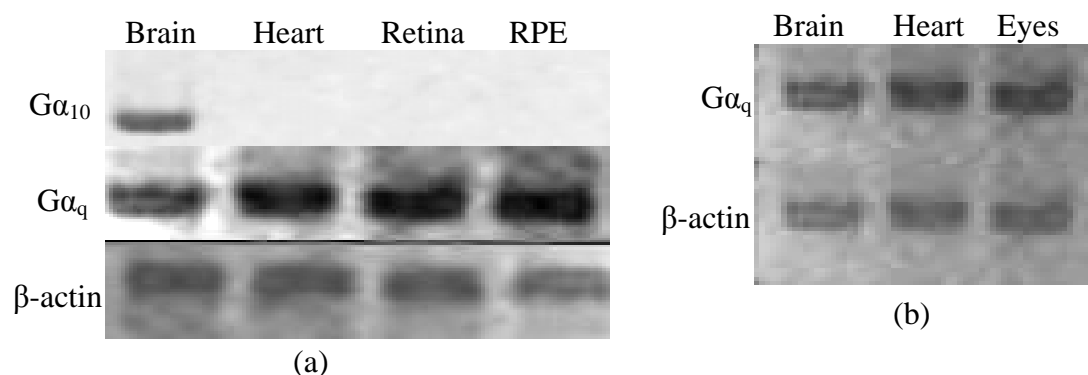bluegill failed to work on zebrafish total RNA.

Figure 16 . RT-PCR results showing expression of $G\alpha_q$ and $G\alpha_{11}$-like ($G\alpha_{10}$) mRNA in bluegill (a) and zebrafish (b). β-actin expression was used as a positive control. The primers used for $G\alpha_q$, $G\alpha_{11}$ and β-actin are given in Table 2.

Immunolocalization of $G\alpha_{q/11}$ in fish eyes

The RT-PCR results showed that $G\alpha_q$ but not $G\alpha_{11}$-like mRNA was detected in

bluegill retina and RPE, suggesting the possibility that G_q-protein, but not the G_{11}-like

protein, might be expressed in retina and RPE. Other $G_{q/11}$ proteins not disclosed in this

study could not be ruled out. To study the $G\alpha_{q/11}$ protein expression in these tissues,

immunolabeling using anti-$G\alpha_{q/11}$ antibody was carried out. The results showed $G\alpha_{q/11}$

immunoreactivity in bluegill choroid, RPE and/photoreceptors (Figure 17). The labeling

observed in the RPE in Figure 17 could be $G\alpha_{q/11}$ labeling in the rod photoreceptors that

are closely interdigitated with the apical processes of RPE. Considerable labeling was

also observed in the retina that was absent in the control (Figure 18). Similar $G\alpha_{q/11}$

labeling was observed in zebrafish eyes (Figure 19).

(a)

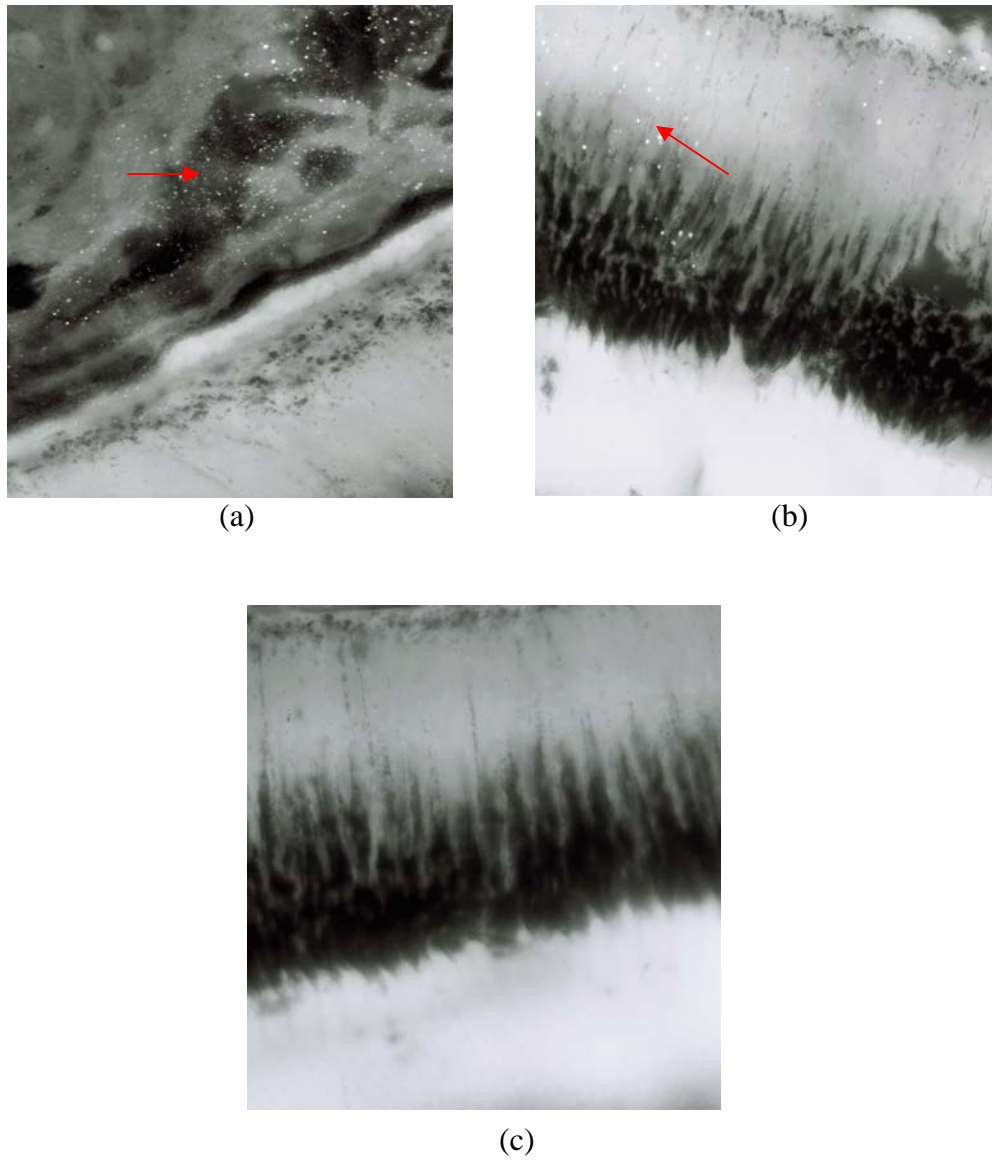

(b)

(c)

Figure 17. Immunolocalization of Gα$_{q/11}$ in bluegill choroid (a) and RPE/photoreceptors (b). The red arrow points to labeling, which appears as specks absent in the control (c), in spite of the high autofluorescence observed.

(a)

(b)

(c)

(d)
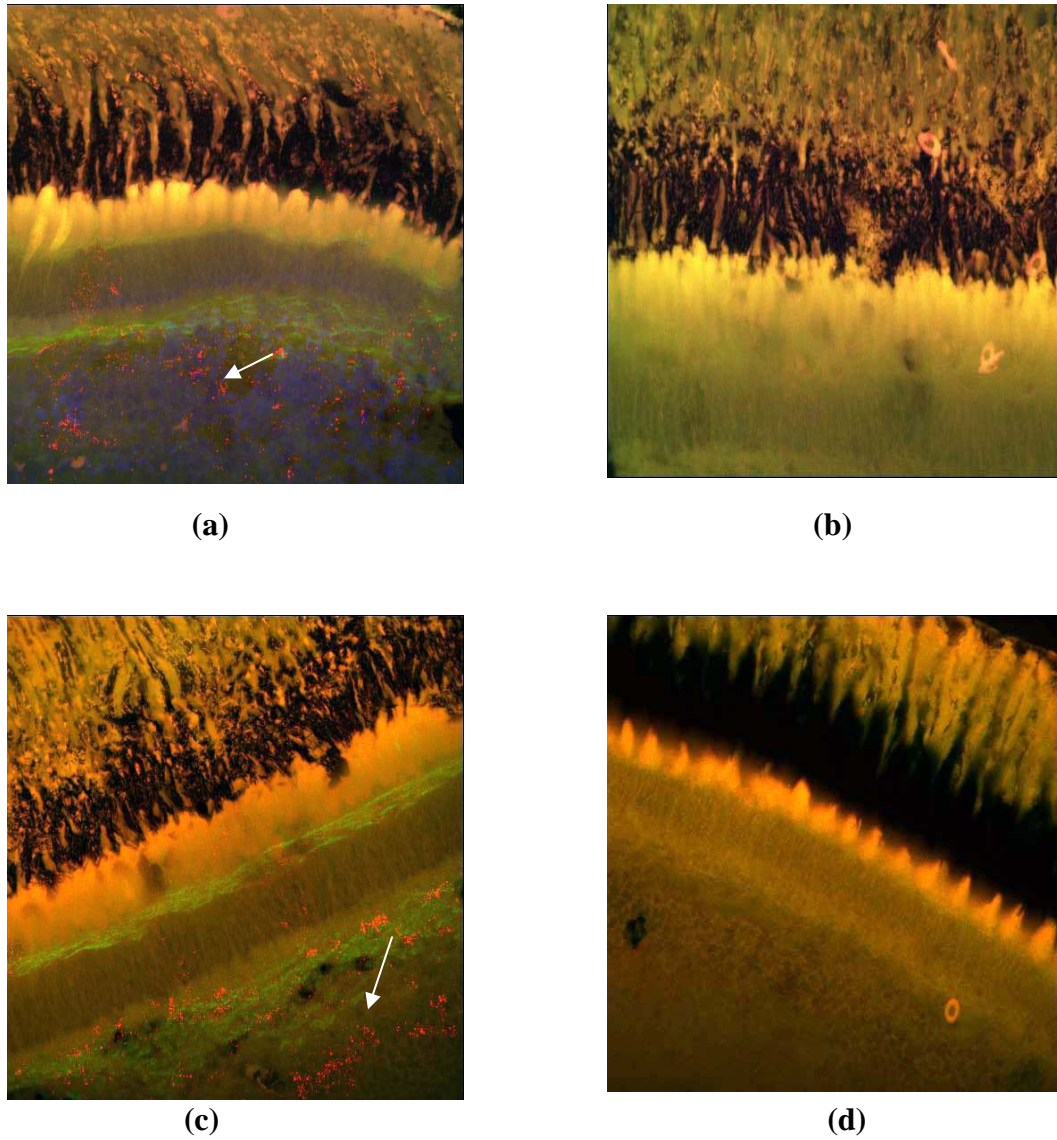
Figure 18. **Immunolocalization of Gα$_{q/11}$ in bluegill retina.** The white arrow points to the Gα$_q$ localization (appearing as red specks) in the retina (a) and (c). F-actin staining (green color) using Alexa Fluor 488- conjugated phalloidin (a and c) and nuclear staining using TOPRO (a) act as positive controls. Controls (b and d) lacking phalloidin and TOPRO treatment show no Gα$_q$ labeling as indicated by the absence of red specks. All images were taken at 60X magnification.

(a)

(b)

(c)

Figure 19. Immunolocalization of Gα$_{q/11}$ in zebrafish RPE/photoreceptors. The red arrows in (a) and (b) show labeling of (appearing as small white specks) in the apical processes of RPE and/or photoreceptors as they are interdigitated with one another (c) represents the control.
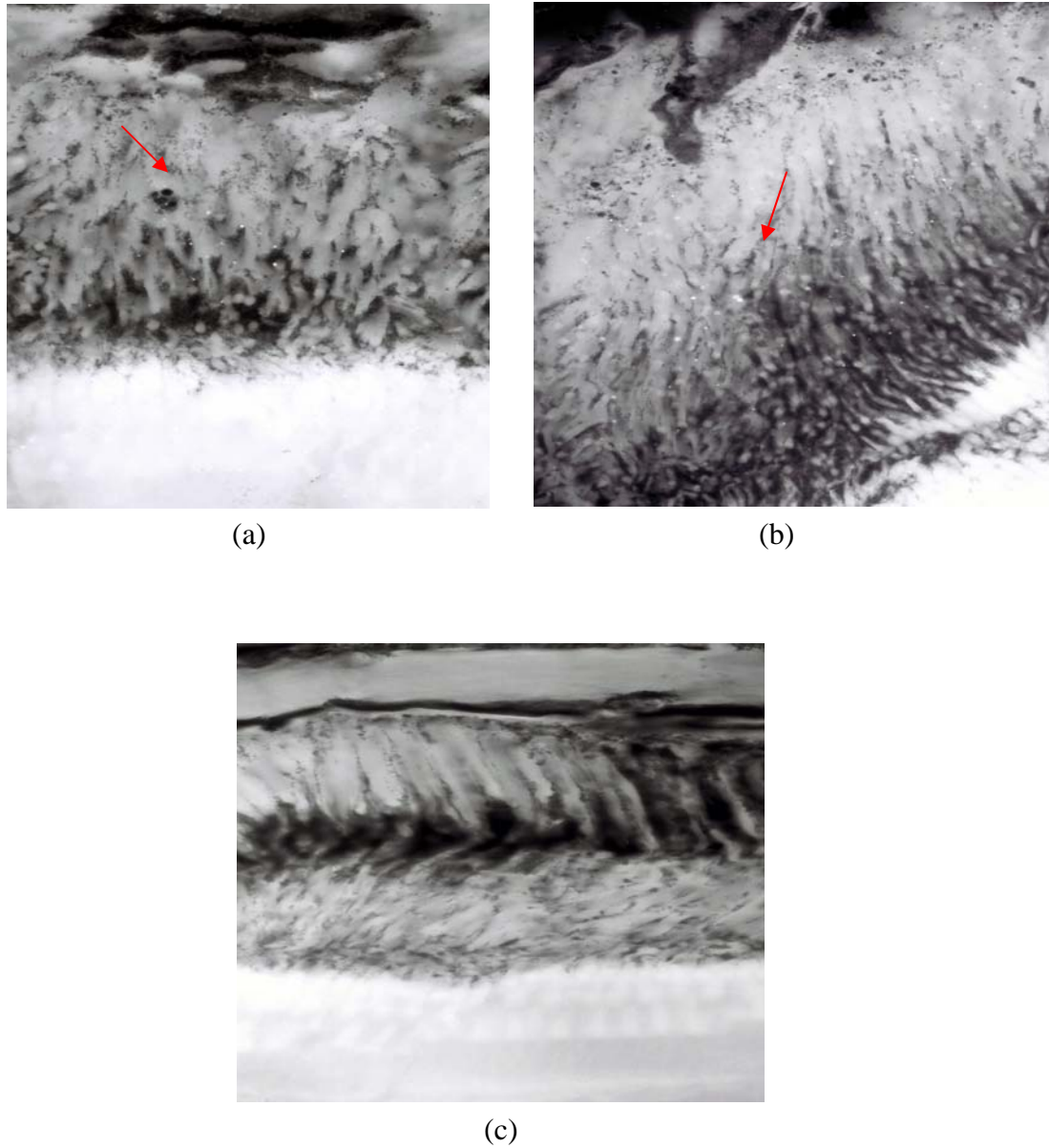
To study whether $G\alpha_{q/11}$ is expressed in fish eyes during development, immunolabeling was carried out for 7 dpf, 14 dpf, 21 dpf, 28 dpf and adult zebrafish eyes. At 7dpf, labeling for $G\alpha_{q/11}$ was observed in eyes though it was difficult to differentiate the specific sites of labeling (Figure 20A and 20B). The control sections did not show the labeling (Figure 20C and 20D). At the metamorphic stage (14dpf), labeling was visible in the retina (Figure 21A) that was absent in the control (Figure 21B). At 21 dpf, $G\alpha_{q/11}$ immunoreactivity was observed distinctly at in the inner retinal layers (Figure 22A, 22B and 22C) and the controls (22D, 22E and 22F) lacked labeling. At 28dpf or the juvenile stage, retina and choroid showed labeling (Figure 23A, 23B and 23C), while the controls lacked labeling (23E, 23F and 23E). In the retina, distinct labeling was observed in the photoreceptor outer segments, the outer and inner nuclear layers and the ganglion cell layer (Figure 23C). At the adult stage (>90dpf), labeling was shown by retina, choroid and photoreceptors and/RPE (Figures 24A, 24B and 24C). The labeling at the RPE could not be differentiated from the photoreceptor labeling as the apical processes of RPE are closely interdigitated with rod photoreceptors.

Figure 20. Immunolabeling of Gα$_{q/11}$ in 7 dpf zebrafish eyes. Labeling of Gα$_{q/11}$ protein in larval (7 dpf) zebrafish eyes. The treatment sections were obtained using (A) 20X and (B) 60X objectives. The white arrow points to labeling seen as red specks. The control sections taken using the (C) 20X, (E) at 60X objectives lack labeling as indicated by the absence of the specks. The scale bars in (A) and (B) represents 100µm and 50µm, respectively.
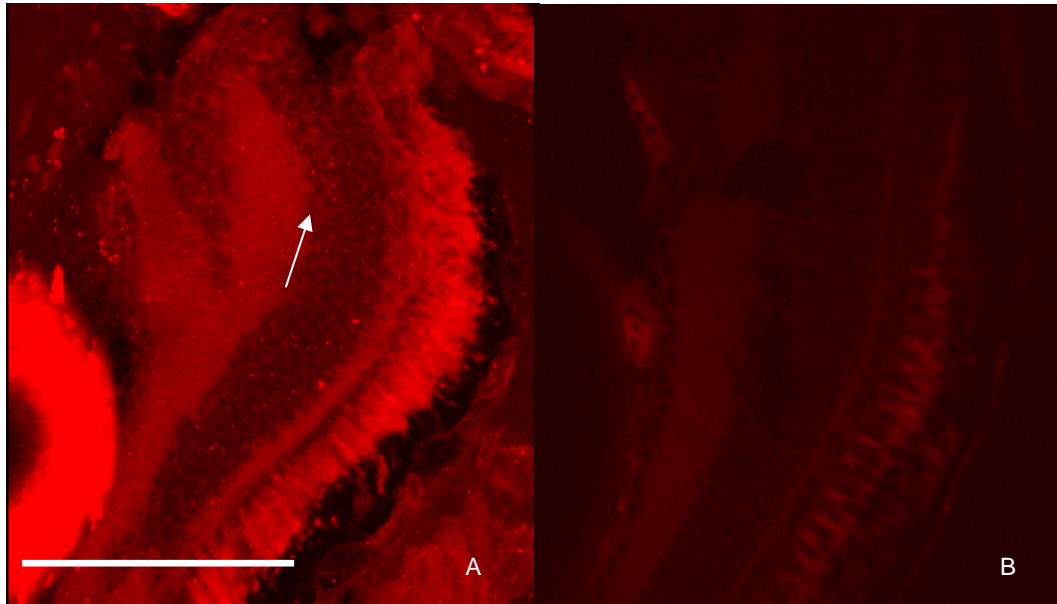
Figure 21. Immunolabeling of Gα$_{q/11}$ in 14 dpf zebrafish eyes. Labeling of Gα$_{q/11}$ protein in 14 dpf zebrafish eyes. The treatment and control sections were obtained using a 60X objective. The white arrow points to labeling seen as red specks, which is absent in control. The scale bar in (A) represents 50µm.
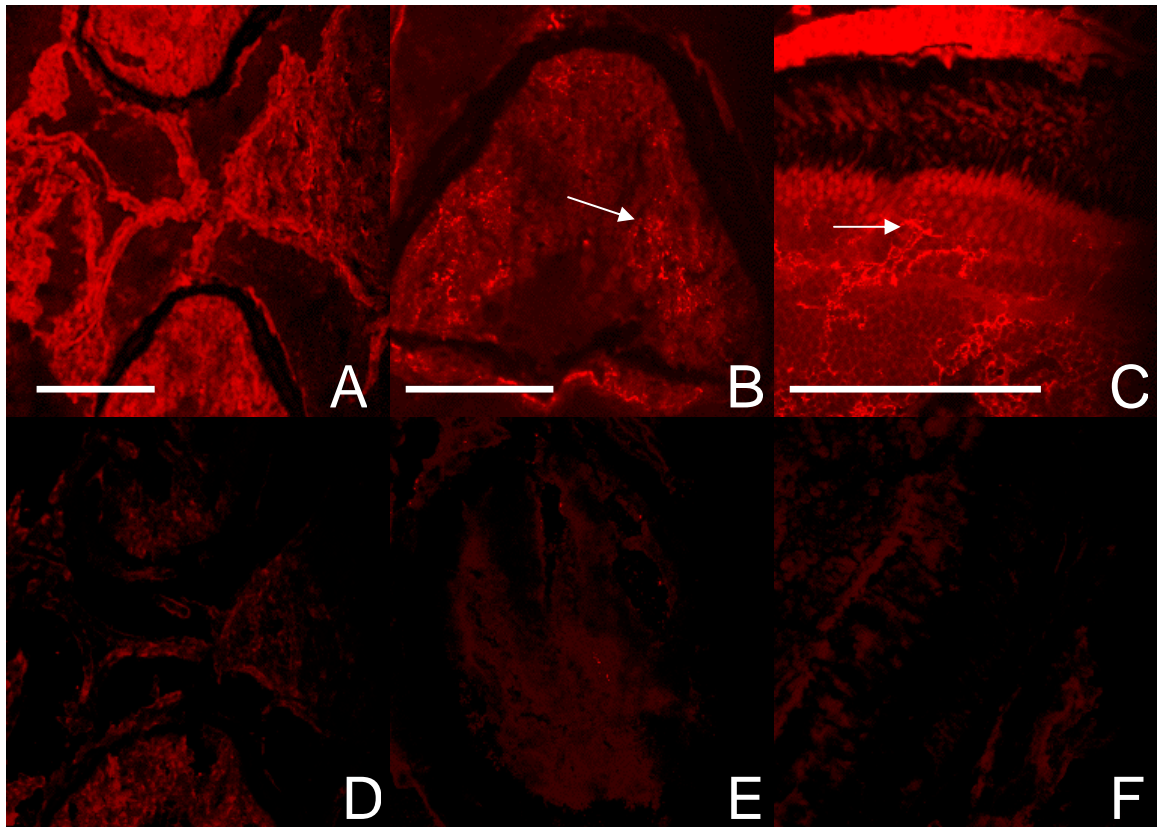
Figure 22. Immunolabeling of Gα q/11 in 21 dpf zebrafish eyes. Labeling of Gαq/11 protein in 21 dpf zebrafish eyes. The treatment sections were obtained using (A) 10X, (B) 20X and (C) 60X objectives. The white arrow points to labeling seen as red specks. The control sections obtained using the (D) 10X, (E) 20X and (F) 60X objectives lack labeling as indicated by the absence of the specks. The scale bars in A, B and C represent 150μm, 100μm and 50μm, respectively.
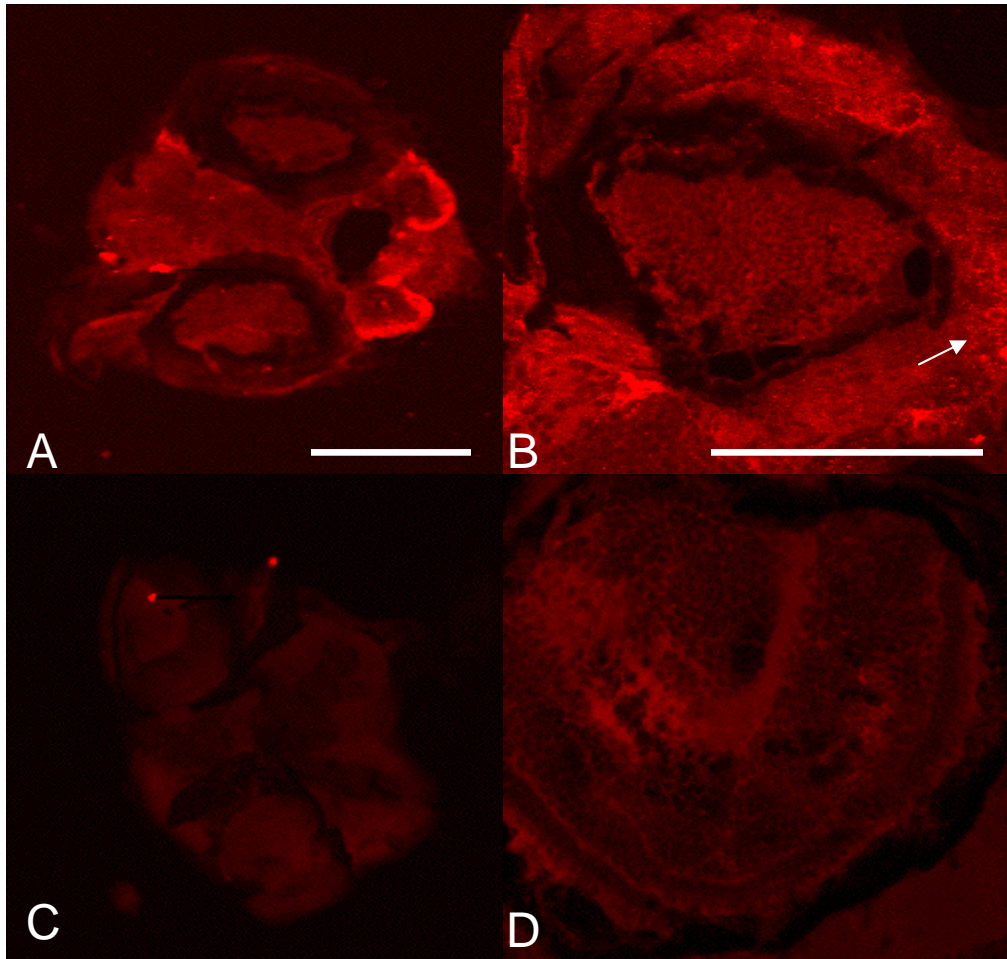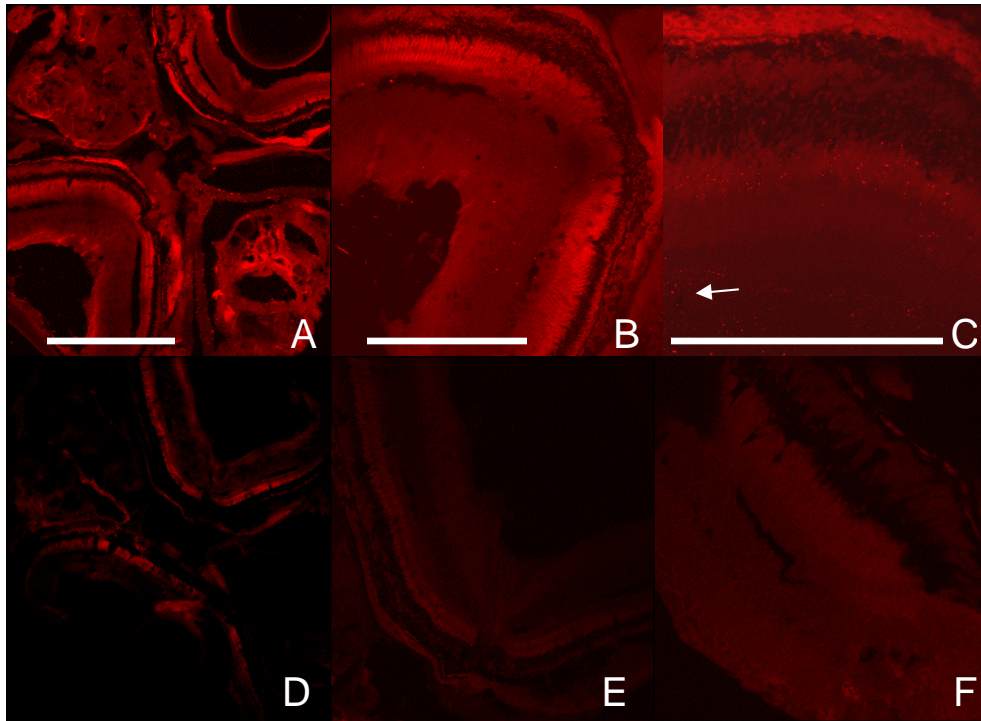
Figure 23. Immunolabeling of Gα$_{q/11}$ in 28 dpf zebrafish eyes. Labeling of Gαq/11 protein in juvenile (28 dpf) zebrafish eyes. The treatment sections were obtained using the (A) 10X, (B) 20X and (C) 60X objectives. The white arrow points labeling seen as red specks. The control sections were obtained using (D) 10X, (E) 20X and (F) 60X objectives and lack labeling as indicated by the absence of the specks. The scale bars in A, B and C represent 150µm, 100µm and 50µm, respectively.

Figure 24. Immunolabeling of Gα$_{q/11}$ in adult zebrafish eyes. Labeling of Gαq/11 protein in adult zebrafish eyes. The treatment sections are (A) at 10X, (B) at 20X and (C) at 60X. The white arrow points labeling seen as red specks. The control sections (D) obtained using 10X, (E) at 20X and (F) at 60X objectives lack labeling as indicated by the absence of the specks. The scale bars in A, B and C represent 150µm, 100µm and 50µm, respectively.
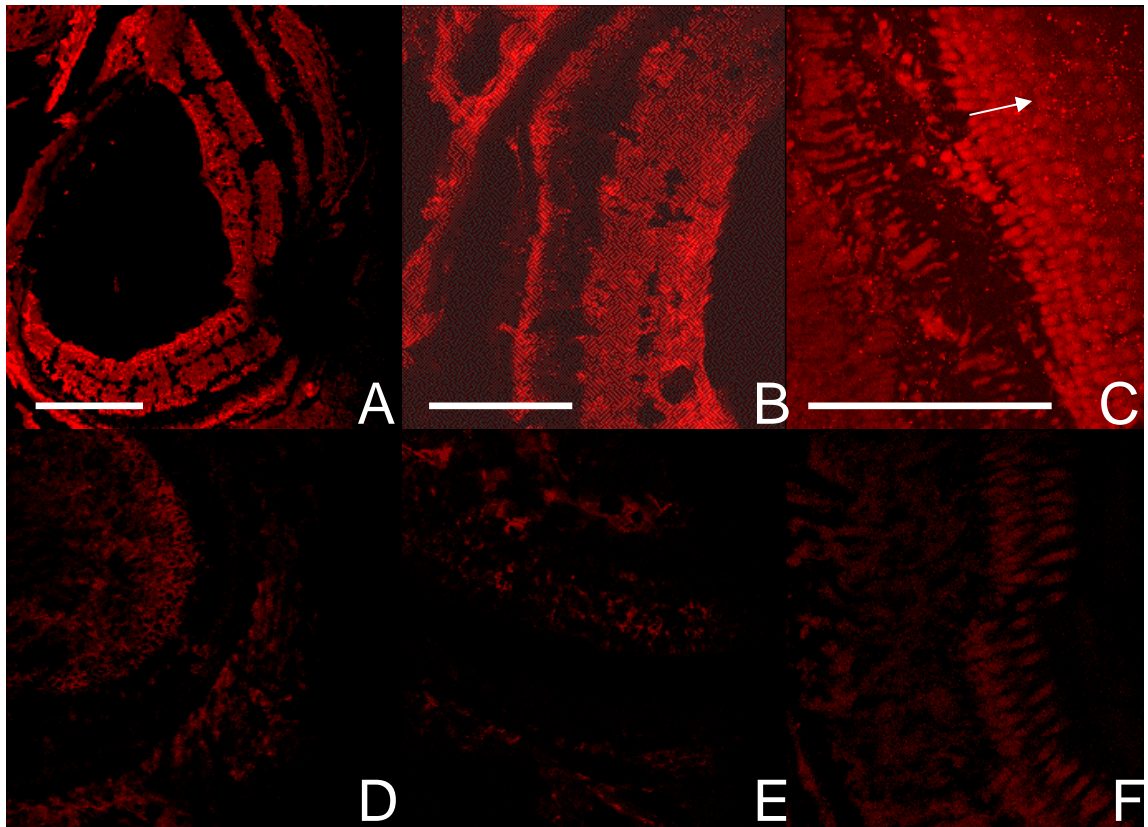
CHAPTER IV

DISCUSSION

The objective of this study was to use a molecular approach to characterize the $G\alpha_q$ and $G\alpha_{11}$ subtypes of G-proteins from bluegill sunfish and to study their expression in fish eyes using reverse transcription polymerase chain reaction and immunohistochemistry. My results indicate that bluegill brain expresses *bona fide* $G\alpha_q$ which is also expressed in retina and RPE. These findings suggest that $G\alpha_q$ could be involved in the signal transduction pathway linking muscarinic receptor activation to pigment granule dispersion. Lastly, the expression of $G\alpha_{q/11}$ was observed in all developmental stages tested in zebrafish, implying that these G-protein subtypes may be involved in the embryonic development of fish.

Molecular characterization of $G\alpha_q$ and $G\alpha_{11}$ from bluegill brain cDNA

I have successfully amplified 692 bp and 893 bp of bluegill $G\alpha_q$ and $G\alpha_{11}$-like gene fragments from brain cDNA, representing ~70% and ~84%, respectively, of the expected sequence, based on comparison with other known vertebrate $G\alpha_q$ and $G\alpha_{11}$ sequences. BLAST searches at the nucleotide and amino acid levels showed that both sequences are similar to their respective vertebrate homologs. They also showed more than 80% similarity to each other. Both sequences showed the critical amino acid

residues and specific domains required for effector activation, receptor coupling and GTPase activity. Phylogenetic analyses confirmed the assignment of the G-protein fragment from bluegill described herein to the $G\alpha_q$ subtype. However, the identity of the fragment retrieved using primers intended to amplify $G\alpha_{11}$ could not be assigned to any of the known $G_{q/11}$ groups with certainty using phylogenetic methods. This gene fragment does not appear to be orthologous to mammalian $G\alpha_{11}$ sequences, but appears to be more closely related to $G\alpha_q$ and $G\alpha_{14.}$ Therefore, it is likely that this $G\alpha_q$-like fragment retrieved from bluegill may be unique in its own way, and I propose that this fragment be referred to as $G\alpha_{10}$. Furthermore, it grouped with zebrafish $G\alpha_{11}$; therefore, I propose that the zebrafish gene also be redesignated as $G\alpha_{10}$. At this juncture, it is perhaps important to add that it is not clear if bluegill or lower vertebrates express $G\alpha_{11,}$ as the primers that were meant to amplify this gene, amplified a different variant ($G\alpha_{10}$), and search of the zebrafish and fugu genomic databases failed to yield any other full length $G_{q/11}$ sequences. Sarwar *et al.* (1996) characterized 14 of the 16 known mammalian homologs of $G\alpha$ multigene family in fugu using a combined approach of low stringency screens of genomic cosmid libraries and PCR amplication using degenerate primers. Though they identified a $G\alpha_q$ and $G\alpha_{11}$ in fugu, the fragments contained only exons 5, 6 and 7, representing only about 30% of the total anticipated sequence. Thus the study reported here is the first one to characterize $G\alpha_{q/11}$-subtypes in fish using sequences representing more than 70% of the coding sequence. Furthermore, because Sarwar *et al.* (1996) failed to include phylogenetic analyses for the assignment of their G-protein subtypes from fugu, our assignment of $G\alpha_q$ from bluegill is more reliable.

Critical amino acids

Amino acids conserved across all Gα subtypes

The alignment of amino acid sequences of Gα subtypes showed that they consist of a GTPase domain and an alpha helical domain. The GTPase domain, common to all GTPases, consists of five alpha helices surrounded by six β strands (Orun, 2006) (underlined areas in Figures 6 and 10). The alpha helical domain is the most divergent of all Gα protein families and is involved in burying the GTP within the core of the protein (Cabrera-Vera *et al*., 2003).The GTPase domain has five distinct motifs involved in GTP binding and hydrolysis (Cabrera-Vera *et al*., 2003) which are marked in gray boxes in Figures 5 and 8. These five motifs are conserved across all Gα protein subtypes and may be divided as G1 or P, G2 or E, G3, G4 and G5 (Sandhya and Vemuri, 1997). The G1 motif in mammals show a consensus sequence GXXXXGK(S/T) at positions 45-53 and are known to interact with GTP and GDP. This region is found in most proteins that bind purine nucleoside triphosphates, like protein kinases and ATPases (Walker *et al*., 1982). The bluegill putative $Gα_q$ fragment characterized herein shows a G1 motif consisting of amino acid residues GTGESGKS, exactly identical in both sequence and position to the G1 motif found in mammals. However, in the bluegill putative $Gα_{10}$ sequence, the residues corresponding to this motif are not known yet as they are encoded 5' to the sequence obtained herein.

The E or G2 motif has a consensus sequence D-(X)n-T. The conserved threonine residue in this motif has been reported to be responsible for Mg^{2+}-binding (Paduch *et al*., 2001), and is located at position 85 in the sequences aligned here. The G2 motif was found in both bluegill $Gα_{10}$ and $Gα_q$ sequences (Figures 6 and 10, respectively), and both

sequences had the necessary threonine residue for binding with Mg^{2+}. In bluegill $G\alpha_{10}$, T^{76} and K^{77} in the G2 motif were replaced by I^{76} and R^{77}, respectively, but bluegill $G\alpha_q$ did not show any such replacement. But as all other residues of the G2 motif in bluegill $G\alpha_{10}$ appears to be identical to the G2 motif in mammalian $G\alpha_{11}$ sequences, the substitution of the two amino acids may be functionally neutral.

The G3 motif in mammals has a consensus sequence DXXG, and this conserved region has also been shown to contribute to GTPase activity (Sandhya and Vemuri, 1997). The G3 motif in both bluegill sequences have the amino acids DVGG at position 205-209, as in mammals (Figures 6 and 10, respectively). The consensus sequence NKXD of the G4 motif interacts with the guanine ring in mammals and stabilizes the guanine nucleotide-binding site (Sprang, 1997). Though this motif was completely conserved in bluegill $G\alpha_{10}$, this region is not yet characterized in bluegill $G\alpha_q$ as this motif is encoded 3' to the sequence obtained herein. The G5 motif consists of the consensus sequence (T/G/C) (C/S)A and acts as a recognition site for the guanine base (Sprang, 1997). This region was conserved in bluegill $G\alpha_{10}$, but the corresponding region has not been characterized in bluegill $G\alpha_q$ yet.

Amino acids that distinguish $G\alpha_{q/11}$ from other $G\alpha$ subtypes

The $G\alpha_q$ and $G\alpha_{11}$ subtypes form a distinct class of G-proteins as they show less than 50% amino acid sequence identity with other $G\alpha$ subtypes. $G\alpha_q$ and $G\alpha_{11}$, when compared with other $G\alpha$ subtypes, exhibit several noteworthy differences, though no unique amino acid replacements were noted (Yokoyama and Starmer, 1992). N-terminal sequences of $G\alpha_q$ and $G\alpha_{11}$ in mammals were reported to differ distinctly from other G-

protein N-terminals due to the presence of methionine residues preceeding the

methionine that was predicted by homology to be the first codon in $G\alpha_q$ and $G\alpha_{11}$

(Strathmann and Simon, 1990). In other words, $G\alpha_q$ and $G\alpha_{11}$ have an additional N-

terminal sequence not seen in other G-protein subtypes. The six amino acids found in

both sequences (MTLESI) are reported to be highly conserved among organisms

suggesting functional significance (Strathmann and Simon, 1990).

In bluegill $G\alpha_q$ and $G\alpha_{10,}$ the N-terminal regions have not been sequenced yet and

could not be compared to other vertebrate sequences. It should perhaps be noted that this

region (MTLESI) is also missing in invertebrate $G\alpha_q$ sequences. The N-termini of some

$G\alpha$ subtypes are subjected to N-myristoylation on a glycine at the second position (Gly-1)

(Buss *et al*., 1987). But Strathmann and Simon (1990) showed that $G\alpha_q$ and $G\alpha_{11}$ differ

from other G-protein subtypes by lacking myristoylation, as their glycine at that position

is replaced by alanine. In bluegill sequences, this replacement could not be confirmed as

the residues at this region are not yet sequenced. The residues at position 46-50 represent

the highly conserved "GAGES-box" motif (GTGES) that has been implicated in GTP-

binding in all G-protein α subunits (Strathmann and Simon, 1990). Mutations at this

region have been shown to affect the GTPase activity of G-proteins (Masters *et al*.,

1989). This region was conserved at the same position in bluegill $G\alpha_q$ (see Figure 10), but

this motif could not be confirmed in bluegill $G\alpha_{10}$ as it has not been fully sequenced yet.

The cysteine residue (position 356) at the C-terminal region of most mammalian G-

proteins can be ADP-ribosylated by pertussis toxin, thereby inactivating the G-protein

(Strahmann and Simon, 1990). Bluegill $G\alpha_{10,}$ like other mammalian $G\alpha_{10}$ sequences, lack

cysteine at position 356. The C^{356} at this position is replaced by Y^{356}. Bluegill $G\alpha_q$

sequence did not include the complete C-terminus and hence this replacement could not be observed (Figure 10). Other G-protein subtypes like $G\alpha_s$, $G\alpha_{olf}$ and $G\alpha_z$ also lack cysteine at this position (Lochrie and Simon, 1988), indicating that these proteins are insensitive to modification induced by pertussis toxin.

Amino acids that differentiate $G\alpha_q$ and $G\alpha_{11}$

The deduced amino acid sequence of $G\alpha_{11}$ in mammals are reported to be 88% identical to that of $G\alpha_q$ and almost all the differences between them are concentrated at the N- terminal half of the protein (Strahmann and Simon, 1990). Out of the 42 amino acid differences among the two subtypes in mouse, 38 are located in the N-terminal region (1-200), and only 4 differences are located in the other half of the protein (201-359). It is also reported that both sequences may be difficult to discriminate using *in vitro* studies as they are about 97% identical at the C-terminal region, the domain that is important for receptor and effector specificity (Strathmann and Simon, 1990). However, Lochrie and Simon (1988) showed that there are strict amino acid sequence differences that identify and differentiate the two subtypes. In bluegill sequences, similar differences in amino acid residues were observed, and the residues that differ between $G\alpha_q$ and $G\alpha_{11}$ in mammalian sequences are highlighted in green in Figures 6 and 10. However, there were several cases in which the $G\alpha_{10}$ sequence had $G\alpha_{11}$-like residues and others where the residues were more $G\alpha_q$-like.

Amino acids critical for receptor coupling

The Gα subunit has specific sites on the sequence that interact with receptors and the best characterized receptor contact region is located at the COOH-terminus (Hamm, 1998). Studies using chimeric Gα subunits suggest that the last five amino acids may contribute to the specificity of receptor G-protein interaction (Hamm, 1998). However, the carboxyl terminal amino acids alone may not be involved in receptor binding. $G\alpha_{oA}$ and $G\alpha_{oB}$, both of which have the same C-terminal amino acids, interact with different receptors (Rens-Domiano and Hamm, 1995). Furthermore, multiple sites in $G\alpha_{16}$ were required for its interaction with C5a receptor (Rens-Domiano and Hamm, 1995). Key residues in receptor coupling have also been identified in the N-terminus and other regions of specific Gα subtypes (Cabrera-Vera *et al*., 2003). The conserved Arg^{340} residue of the DRY motif on G-protein coupled receptors (GPCRs) is reported to be an important factor in the receptor sequence leading to GDP release and Gα activation (Oliveira *et al*., 1993).

Furthermore, Oliveira *et al*. (1999) suggested that the binding site of Arg^{340} on the GPCR could be either Asp^{333} or Asp^{337} in the α5 helix of the G-protein. This possibility suggests that receptor-Gα binding specificity may not be controlled by one specific domain, but could be the result from a network of contacts between the two and may differ for each Gα subunit and receptor, resulting in a large number of possible combinations of G-protein-receptor interactions. In bluegill $G\alpha_{11}$-like fragment, the C-terminal regions are similar to mammalian $G\alpha_{11}$ sequences but the $G\alpha_q$ fragment retrieved from bluegill is incompletely sequenced in the C-terminus and hence its identity with respect to its receptor-coupling motif could not be confirmed.

Amino acids critical for binding to effector (PLC)

The sites of interaction between Gα subunit and its effectors are still not fully understood. The alpha helical domain has shown to be important for the specificity of the effector-coupling to G-proteins (Rens-Domiano and Hamm, 1995). Berlot and Bourne (1992) have shown that three discrete regions are important for the interaction of Gα with specific effector proteins like adenylyl cyclase or phospholipase C. These regions correspond to the residues 236-240 (region I), 276-285 (region II) and 349-356 (region III) in $G\alpha_s$ subtype. In $G\alpha_t$, at least two such putative regions of effector interaction have been identified (Rens-Domiano and Hamm, 1995).

Helper *et al*. (1995) demonstrated that Cys^9 and Cys^{10} (with or without palmitoylation) are required for the activation of PLC-β1 in $G\alpha_q$. They also showed that these residues are important for interaction with the receptor to a certain extent and that the amino terminus of $G\alpha_q$ conveys a hydrophobic character to the protein distinct from that contributed by palmitate. Arkinstall *et al*. (1995) used multiple overlapping synthetic peptides to map regions of $G\alpha_q$ that react with recombinant PLC-β1. With this approach they identified two distinct regions of $G\alpha_q$ (Ser^{251}-Gln^{256} and Ala^{306}-Asp^{319}) that inhibited $G\alpha_q$-mediated PLC-activation. Venkatakrishnan and Exton (1995) on the other hand, used transient expression of chimeras and measurement of inositol phosphate production in HEK-293 cells to reveal that Ile^{217}-Lys^{276} contained the PLC activation site. Alanine scanning mutagenesis of this region also helped them narrow down two clusters of amino acid residues (Asp^{243}, Asn^{244}, Glu^{245} and Arg^{256}, Thr^{257}) that are specifically required for PLC interaction (highlighted in yellow in Figures 6 and 10). While both bluegill sequences showed the amino acid residues 243-245 needed for PLC activation, the

residues 256-257 could not be confirmed in $G\alpha_q$ as this region is still not sequenced

(Figure 10).

Amino acids critical for binding with βγ dimer

The amino terminus of Gα subunit has been shown to be critical for its interaction

with the βγ dimer. The specific residues of the amino terminus involved in interaction

with $G_{\beta\gamma}$ may vary among and within each Gα family. For example, residues 7-11 on the

$G\alpha_o$ subtype were shown to be critical for interaction with $G_{\beta\gamma}$ (Rens-Domiano and

Hamm, 1995), but for $G\alpha_s$, the residues at this position were not critical for binding with

βγ-subunit. Another possible βγ-binding site on Gα sequence could be the switch regions

as GTP binding decreases $G_{\beta\gamma}$ affinity (Rens-Domiano and Hamm, 1995). The exact βγ-

binding site on $G\alpha_{q/11}$ has not been identified yet though it has been speculated that the

binding site is located in the amino terminus of the sequence (Rens-Domiano and Hamm,

1995).

Phylogeny and evolution of G-proteins

A number of different mechanisms like tandem gene duplication, genome

duplication or tetraploidization and reverse transcription (resulting in pseudogene

formation) have been implicated in the development of multigene families (Wilkie *et al*.,

1992; Wilkie and Yokoyama, 1992). There is also evidence that suggests that the

eukaryotic genome has undergone multiple genome duplication events and that the most

recent event occurred approximately three hundred million years ago (Wilkie *et al*.,

1992). The phylogeny of Gα multigene family in mammals have shown that $G\alpha_q$ and $G\alpha_i$

subtypes each form single monophyletic units, suggesting that they are not closely related to one another. Previous studies have also shown that the $G\alpha_{12/13}$ family is closely related to the $G\alpha_{q/11}$ family and that their common ancestor diverged from the $G\alpha_s$ family (Wilkie *et al*., 1992; Wilkie and Yokoyama, 1994).

The topology of all trees generated in the study reported herein (which includes fish and amphibian G-protein sequences) showed a slightly different grouping of G-protein subtypes. All tree topologies identified the two genes characterized in the current study as belonging to the $G\alpha_{q/11}$ family based on the available nomenclature from mammalian sequences. However, one noteworthy difference in the trees reported herein is that the bluegill $G\alpha_{10}$ (LmG11), zebrafish predicted $G\alpha_{11}$ (DrG11) and frog $G\alpha_{11}$ (XlG11) are clustered separately from the mammalian $G\alpha_{11}$ sequences, and they appear to be more closely related to $G\alpha_q$ and $G\alpha_{14}$ than they are to mammalian $G\alpha_{11}$. This result suggests that the lower vertebrate $G\alpha_{11}$ sequences are unique in their own way, and I propose they should be renamed (for example, as $G\alpha_{10,}$ due to their shared ancestory with $G\alpha_q$ and $G\alpha_{14}$). Bluegill $G\alpha_q$ formed a sister clade to the mammalian $G\alpha_q$ sequences but as all $G\alpha_q$ sequences were monophyletic, the bluegill $G\alpha_q$ fragment can be considered as *bona fide* $G\alpha_q$. The bluegill $G\alpha_i$ (sequenced by Sandra Becerra) may be named as $G\alpha_{i1/i3}$ as it is more closely related to $G\alpha_{i1}$ and $G\alpha_{i3}$ than to $G\alpha_{i2}$.

In the phylogenetic analyses conducted in this study, the grouping of mammalian G-protein subtypes appears to be similar to the G-protein phylogenies put forth by Yokoyama and Starmer (1992) and Wilkie *et al.* (1992). However, they are drastically different from studies by Simon *et al.* (1991) and Strathmann and Simon (1991). In their studies, they reported that $G\alpha_q$ and $G\alpha_i$ are closely related to one another. This difference

in inferences about G-protein phylogeny could be due to the lack of an outgroup in their studies or to their assumption of a constant rate of amino acid replacement in their analysis. Previous research has shown that the nucleotides at the third codon positions evolve faster than those at the first or second codon positions (Felsenstein, 1978) and hence should be given a lower weight during phylogenetic analysis. Similarly, the rate of transition is much higher than the rate of transversion (Collins *et al*., 1994), and should be down-weighted while conducting parsimony analysis to make an accurate phylogenetic inference. Neither Simon *et al.* (1991) nor Strathmann and Simon (1991) down-weighted the nucleotides of their dataset at the third codon positions, and both studies failed to down-weight the nucleotide transitions, calling their results into question.

The evolution of Gα multigene family has been explained by a simple model in mammals. Mammalian $G\alpha_{11}$ and $G\alpha_{15}$ are closely related as are $G\alpha_q$ and $G\alpha_{14}$. Previous mapping studies suggest that a single progenitor gene was tandemly duplicated giving rise to $G\alpha_q$ and $G\alpha_{11}$ (Wilkie *et al*., 1992). These genes were duplicated again onto another chromosome, giving rise to four Gq classes. This hypothesis was supported by the fact that on mouse chromosome 10, the genes for $G\alpha_{11}$ and $G\alpha_{15}$ were tandemly duplicated in a head-to-tail array (Davignon *et al*., 1996). Their gene structure showed that the coding sequence of each gene was contained in seven exons, and the two genes together spanned about 43 kb with 6 kb of intergenic region. Moreover, $G\alpha_q$ and $G\alpha_{14}$ were found to cosegregate on chromosome 19 in mouse (Yokoyama and Starmer, 1992; Davignon *et al*., 1996). In the tree topologies generated in the current study, mammalian, $G\alpha_q$ and $G\alpha_{14}$ were closely related as were mammalian $G\alpha_{11}$ and $G\alpha_{15}$.

Previous findings suggest that $G\alpha_i$, $G\alpha_o$ and $G\alpha_t$ genes may be descendents of a common tandemly duplicated progenitor (Wilkie *et al*., 1992; Wilkie and Yokoyama, 1994). In their tree topology, $G\alpha_z$ was observed to be the most divergent among Gi class of proteins, and it also mapped separately from all other $G\alpha$ genes in other studies (Yokoyama and Stamer, 1992; Wilke *et al*., 1992). The intron organization of mammalian $G\alpha_z$ was shown to be unique with an additional intron in the 5' untranslated region (Kaziro *et al*., 1991). Wilkie *et al.* (1992) inferred based on its gene structure that $G\alpha_z$ resembled an incomplete pseudogene that may have arisen from a reverse transcription event. They also speculated, based on its highly conserved amino acid sequence in rats and humans, that this gene could have been recruited as a functional gene prior to the divergence of rodents and primates (Wilkie *et al*., 1992). In the study reported herein, $G\alpha_z$ formed a sister taxon with $G\alpha_o$, and $G\alpha_t$ formed a separate, highly divergent group within the G_i ingroup.

$G\alpha_q$ and $G\alpha_{11}$ localization in fish tissues

Though $G\alpha_q$ and $G\alpha_{11}$ have been reported to be expressed ubiquitously in all tissues (Strathmann and Simon, 1990; Wettschureck and Offermanns, 2005), numerous studies have independently illustrated that despite their high similarity, there are some tissues where only one subtype is expressed. For example, human and dog platelets express only $G\alpha_q$ but not $G\alpha_{11}$ (Milligan and Mitchell, 1993; Ushikubi *et al*., 1994). Another study, particularly relevant to the results reported herein, showed that $G\alpha_{11}$ is the only G-protein besides transducin ($G\alpha_t$) identified in the rod outer segments of bovine retina (Peng *et al*., 1997). They also noted that $G\alpha_q$ expression was detected in the inner

retinal layers. My results using RT-PCR show that $G\alpha_q$, but not $G\alpha_{10}$, mRNA is expressed

in bluegill RPE and retina. Immunolocalization suggests $G\alpha_{q/11}$ immunoreactivity in the

photoreceptors, but the rod photoreceptors are so tightly interdigitated with the RPE that

the labeling cannot be distinguished between the apical processes of the RPE and the

photoreceptor outer segments using confocal microscopy. Electron microscopy would be

more effective in distinguishing the labeling between the RPE and the photoreceptor

outer segments.

The expression profile of $G_{q/11}$ in chick developing retina showed that this

subtype is expressed from early embryonic stage and their levels did not change during

later stages of development (Shah and Hausman, 1996). However, in developing retina

from rat, $G\alpha$ distribution ($G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_o$) differed, suggesting that each subtype may

have a unique function at various stages of development (Oguni *et al*., 1996). In zebrafish

eye development reported here, the $G\alpha_{q/11}$ immunoreactivity was observed at the larval

(7dpf), metamorphic (14 dpf and 21 dpf), juvenile (28 dpf) and adult stages. In all stages,

$G\alpha_{q/11}$ expression was detected in the retina, though the differentiation of labeling in the

photoreceptor outer segments, outer nuclear layer and inner nuclear nuclear layers were

evident only after the juvenile stages. Though the levels of expression at different stages

were not determined, it is clear that $G\alpha_q$ does play a role in the proper development of

fish eyes.

Future studies

My role in our laboratory's overall research was to elucidate the G-protein

subtype that may be involved in carbachol-induced pigment granule dispersion in bluegill

RPE. My results indicate that $G\alpha_q$ is expressed in RPE, and therefore could be the G-protein subtype involved in transducing the signal from the muscarinic receptor to the effector, producing a cascade of events that lead to pigment granule dispersion in fish eyes. However, as the primers designed to retrieve $G\alpha_{11}$ amplified a novel sequence ($G\alpha_{10}$), the next step should be to check whether fish express $G\alpha_{11}$ like mammals and to check if this geneis expressed in RPE like $G\alpha_q$. Furthermore,studies using techniques like RNA interference (siRNA) that can knock down specific G-protein subtypes in fish RPE will help demonstrate their role in pigment movements. More knockout studies on downstream signaling components like PLC, IP3, DAG, PKC and PDE will also help elucidate whether the model of signal transduction suggested by Phatarpekar *et al*. (2005) is supported.

Another important study that I would recommend for the future is to look into the expression of $G\alpha_q$ and/or $G\alpha_{11}$ in rod outer segments of bluegill. For the last couple of years, many studies have reported the presence of a phosphoinositide pathway in vertebrate photoreceptor (especially rod) outer segments. Despite these findings, the components involved in the pathway were unclear until Peng *et al*. (1997) identified $G\alpha_{11}$ subtype as the only G-protein besides transducin in rod outer segments of bovine retina. They also identified PLC $\beta4$ in rod outer segments. My RT-PCR results in bluegill retina suggest that there is a high possibility of $G\alpha_q$ expression in bluegill rod outer segments. Thus, future studies on fish rods may raise the interesting possibility that they differ from other vertebrates in expressing $G\alpha_q$ but not $G\alpha_{11}$ in their retinal photoreceptors. Another obvious question that comes out of this study then would be whether $G\alpha_q$ and $G\alpha_{11}$ bear any function other than phototransduction in this location.

Lastly, it would be interesting to evaluate the differences in the expression of genes encoding all G-protein subtypes between dark adapted and light adapted fish RPE using microarray analysis. Microarrays may also be used to study expression levels of muscarinic receptors, G-proteins and other signaling components during fish eye development.

REFERENCES

Allegeier, A., S. Offermanns, I. Van Sande, K. Spicher, G. Schultz, J. E. Dumont. 1994. The human thyrotropin receptor activates G-proteins Gs and Gq/11. *J Biol. Chem.* 269(19):12733-13735.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic Local Alignment Search Tool. *J. Mol. Biol.* 215:403-410.

Arkinstall, S., M. Edgerton, M. Payton, and K. Maundrell. 1995. Coexpression of the neurokinin NK2 receptor and G-protein components in the fission yeast *Schizosaccharomyces pombe. FEBS Lett.* 375:183-187.

Arenzana, F. J., D. Clemente, R. Sanchez-Gonzalez, A. Porteros, J. Aijon, and R. Arevalo. 2005. Development of the cholinergic system in the brain and retina of the zebrafish. *Brain. Res. Bull*. 66:421-425.

Ball A. K.,W. H. Baldridge, and T. C. Fernback. 1993. Neuromodulation of pigment movement in the RPE of normal and 6-OHDA-lesioned goldfish retinas. *Vis Neurosci.* 10:529–540.

Berlot, C. H. and H. R. Bourne. Identification of effector activating residues of Gs alpha. 1992. *Cell* 68:911-922.

Bilofsky, H. S. and C. Burks. 1988. The GenBank genetic sequence data bank. *Nucleic acids Res*. 16:1861-1864.

Birnbaumer, L. 1990. G proteins in signal transduction. *Annu Rev Pharmacol Toxicol*. 30:675-705.

Bourne, H. R., D. A. Sanders, and F. McCorkmick. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* 348: 125-132.

Broadley, K. J. and D. R. Kelly. 2001. Muscarinic receptor agonists and antagonists. *Molecules* 6:142-193.

Burnside, B. 2001. Light and circadian regulation of retinomotor movement. *Prog. Brain Res.* 131:477-485.

Burnside, B. and B. W. Nagle. 1983. Retinomotor movements of photoreceptors and retinal pigment epithelium: mechanisms and regulation. *Progress in Retinal Research (Edited by: Osborne N and Chader G).* New York, Pergamon Press, 2:67-109.

Buss, J. E., S. M. Mumby, P. J. Casey, A. G. Gilman, and B. M. Sefton. 1987. Myristoylated α-subunits of Guanine Nucleotide binding Regulatory Proteins. *Proc. Natl. Acad. Sci. U. S. A.* 84:7493-7497.

Cabrera-Vera, T. M., J. Vanhauwe, T. O. Thomas, M. Medkova, A. Preininger, M. R. Mazzoni, and H. E. Hamm. 2003. Insights into G protein structure, function, and regulation. *Endocrine Reviews* 24(6):765-781.

Caulfield, M. P., and N. J. Birdsall. 1998. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev.* 50:279-90.

Cavallaro, B. and B. Burnside. 1988. Prostaglandins E1, E2, and D2 induce dark-adaptive retinomotor movements in teleost retinal cones and RPE. *Invest. Ophthalmol. Vis. Sci.* 29:882-91.

Collins, T. M., P. H. Wimberger, and G. J. P. Naylor. 1994. Compositional bias, character-state bias, and character-state reconstruction using parsimony. *Syst. Biol.* 43:482-496.

Das, N., J. Majumder, and U. B. DasGupta. 2000. Ras gene mutations in oral cancer in eastern India. *Oral Oncol.* 36(1):76-80.

Davignon, I., M. Barnard, O. Gavrilova, K. Sweet, and T. M. Wilkie. 1996. Gene structure of Murine Gna11 and Gna15: Tandemly duplicated Gq class G protein α subunit genes. *Genomics* 31:359-366.

Dearry, M. and B. Burnside. 1986. Dopaminergic regulation of cone retinomotor movement in isolated teleost retinas. I. Induction of cone contraction is mediated by D2 receptors. *J. Neurochem.* 46:1006-1021.

Dearry, M. and B. Burnside. 1988. Stimulation of distinct D2 dopaminergic and α2-adrenergic receptors induces light-adaptive pigment dispersion in teleost retinal pigment epithelium. *J. Neurochem.* 51:1516-1523.

Douglas, R. H. 1982. The function of photomechanical movements in the retina of the rainbow trout (*Salmo gairdneri*). *J Exp. Biol.* 96:389-403.

Farber, D. B., D. W. Souza, D. G. Chase, and R. N. Lolley. 1981. Cyclic nucleotides of cone-dominant retinas: Reduction of cyclic AMP levels by light and by cone degeneration. *Invest. Ophthalmol. Vis. Sci.* 20(1):24-31.

Felsenstein, J. 1978. Cases in which parsimony or compatibility methods will be positively misleading. *Syst. Zool.* 27:401-410.

García, D. M. 1998. Carbachol-induced pigment granule dispersion in teleost RPE. *Cytobios*. 94:31-37.

García, D. M. and B. Burnside. 1994. Suppression of cAMP-induced pigment granule aggregation in RPE by organic anion transport inhibitors. *Invest. Ophthalmol. Vis. Sci.* 35:178-88.

GeneCodes. 2003. Sequencher. v4.2. Ann Arbor, Michigan, USA.

Gilman, A. G. 1987. G-proteins: Transducers of receptor-generated signals. *Annu. Rev. Biochem*. 56:615-650.

Go, L. and J. Mitchell. 2003. Palmitoylation is required for membrane association of activated but not active invertebrate visual Gqα. *Comp. Biochem. and Phys. Part B.* 135:601-609.

González, A., III, E. L. Crittenden, and D. M. García. 2004. Activation of muscarinic acetylcholine receptors elicits pigment granule dispersion in retinal pigment epithelium isolated from bluegill. *BMC Neurosci*. 5:23.

Gosens, R., D. Schaafsma, S. F. Nelemans, A. J. Halayko. 2006. Rho-Kinase as a Drug Target for the Treatment of Airway Hyperresponsiveness in Asthma. *Mini Rev. Med. Chem.* 6(3):339-348.

Hamm, H. E. 1998. The many faces of G-protein signaling. *J. Biol. Chem*. 273:669-673.

Hayashi, H. and R. Fujii. 1994. Pharmacological profiles of the subtypes of muscarinic cholinoreceptors that mediate aggregation of pigment in the melanophores of two species of catfish. *Pig. Cell. Res.* 7(3):175-183.

Hepler, J. R., G. H. Biddlecome, C. Kleuss, L. A. Camp, S. L. Hofmann, E. M. Ross, and A. G. Gilman. 1995. Functional importance of the amino terminus of Gqα. *J. Biol. Chem.* 271:496-504.

Hickson, R. E., C. Simon, and S. W. Perrey. 2000. The performance of several multiple – sequence alignment programs in relation to secondary structure features for an rRNA sequence. *Mol. Biol. Evol.* 17:530-539.

Hood, D. C. and D. G. Birch. 1993. Light adaptation of human rod photoreceptors: the leading edge of human a-wave and models of rod receptor activity. *Vision Res.* 33(12):1605-1618.

Hsieh, D. J., and C. F. Liao. 2002. Zebrafish M2 muscarinic acetylcholine receptor: cloning, pharmacological characterization, expression patterns and roles in embryonic bradycardia. *Br. J. Pharmacol*. 137:782-92.

Huelsenbeck, J. P., F. Ronquist, R. Nielson, and J. P. Bollback. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* 294:2310-2314.

Iuvone, P. M., G. Tocini, N. Pozdeyev, R. Haque, D. C. Klein and S. S. Chaurasia. 2005. Circadian clocks, clock networks, arylalkaylamine N-acetyltransferase, and melatonin in the retina. *Progress in Retinal and Eye Research* 24:433-456.

Kasahara, T., T. Okano, T. Haga, and Y. Fukada. 2002. Opsin-G11-mediated signaling pathway for photic entrainment of the chicken pineal circadian clock. *J. Neuroscience* 22:7321-7325.

Kaziro,Y., H. Itoh, T. Kozasa, M. Nakafuku, and T. Satoh. 1991. Structure and function of signal-transducing GTP-binding proteins. *Annu. Rev. Biochem.* 60:349-400.

Keith, T. A., V. Radhakrishnan, S. Moredock, and D. M. Garcia. 2006. Uptake of ^{3}H-cAMP by retinal pigment epithelium isolated from bluegill sunfish (*Lepomis macrochirus*). *BMC Neurosci*. 7:82.

Kelly, J. F., K. Storie, C. Skamra, J. Bienias, T. Beck, and D. A. Bennett. 2005. Relationship between Alzheimer's disease clinical stage and $G_{q/11}$ in subcellular fractions of frontal cortex. *J. Neural. Trans*. 112(8):1435-1463.

King-Smith, C., P. Paz, C. W. Lee, W. Lam, and B. Burnside. 1997. Bidirectional pigment granule migration in isolated retinal pigment epithelial cells requires actin but not microtubules. *Cell Motil. Cytoskeleton* 38(3):229-249.

Kuhn, B., A. Schmid, C. Harteneck, T. Gudermann, and G. Schultz. 1996. G proteins of the Gq family couple the H2 histamine receptor to phospholipase C. *Mol. Endocrinol*. 10:1697-1707.

Lochrie, M. A. and M. I. Simon. 1988. G protein multiplicity in eukaryotic signal transduction systems. *Biochemistry* 27(14):4957-4965.

Maddison, D. R. and W. P. Maddison. 2001. MacClade 4: Analysis of phylogeny and character evolution. Version. 4.02. Sinauer Associates, Sunderland, Massachusetts.

Masters, S. B., R. T. Miller, M. H. Chi, F. H. Chang, B. Beiderman, N. G. Lopez, and H. R. Bourne. 1989. Mutations in the GTP-binding site of Gs alpha alter stimulation of adenylyl cyclase. *J. Biol. Chem*. 264(26):15467-15474.

Mangel, S. C. and J. E. Dowling. 1987. The interplexiform-horizontal system of fish retina: effects of dopamine, light stimulation and time in the dark. *Proc. R. Soc. Lond. B Biol. Sci*. 231:91-121.

McCormack, C. A. and B. Burnside. 1991. Effects of circadian phase on cone retinomotor movements in the Midas cichlid. *Exp. Eye. Res.* 52:431-438.

Milligan, G. and F. M. Mitchell. 1993. An arginine residue is the site of receptor-stimulated, cholera toxin-catalysed ADP-ribosylation of pertussis toxin-sensitive G-proteins. *Cell Signal*. 5:485-93.

Neer, E. J. 1995. Heterotrimeric G proteins: Organizers of transmembrane signals. *Cell* 80(2):249-257.

Nürnberg, B., K. Spicher, R. Harhammer, A. Bosserhoff, R. Frank, H. Hilz and G. Schultz. 1994. Purification of a novel G-protein alpha o subtype from mammalian brain. *Biochem. J.* 300:387-394.

Offermanns, S., L. Zhao, A. Gohla, I. Sarosi, M. I. Simon, and T. M. Wilkie. 1998. Embryonic cardiomyocte hypoplasia and craniofacial defects in $G\alpha_q$- $G\alpha_{11}$-mutant mice. *The EMBO J. 17*:4304-4312.

Offermanns, S. and M. I. Simon. 1995. $G\alpha15$ and $G\alpha16$ couple a wide variety of receptors to phospholipase C. *J. Biol. Chem.* 270(25):15175-15180.

Oguni, M., H. Shinohara, T. Asano, K. Kato, and T. Setogawa. 1996. Ontogeny of GTP-binding proteins, Gi and Go, in rat retina. *Histochem. and Cell. Biol.* 106(2):235-240.

Oliveira, L., A. C. M. Paiva, and G. Vriend. 1993. A common motif in G-protein coupled seven transmembrane helix receptors. *J. Comp.-Aid. Mol. Des.* 7:649-658.

Oliveira, L., A. C. M. Paiva, and G. Vriend. 1999. A low resolution model for the interaction of G-protein with G-protein coupled receptors. *Protein Engineering* 12:1087-1095.

Orun, O. 2006. A structural approach to G-protein signaling mechanisms:α-subunits. *Marmara Medical Journal* 19(1):41-45.

Paduch, M., F. Jelen, and J. Otlewski. 2001. Structure of small G proteins and their regulators. *Acta Biochimica Polonica* 48(4):829-850.

Peng, Y.W., S. G. Rhee, W. P. Yu, Y. K. Ho, T. Schoen, G. J. Chaden, and K. W. Yau. 1997. Identification of components of a phosphoinositide signaling pathway in retinal rod outer segments. *Proc. Natl. Acad. Sci. U. S. A.* 94:1995-2000.

Pearson, W. R. 1990. Rapid and Sensitive Sequence Comparison with FASTP and FASTA. *Methods in Enzymology* 183:63-98.

Phatarpekar, P. V., S. F. Durdan, C. M. Copeland, E. L. Crittenden, J. D. Neece, and D. M. Garcia. 2005. Molecular and pharmacological characterization of muscarinic receptors in retinal pigment epithelium: role in light-adaptive pigment movements. *J. Neurochem*. 95:1504-20.

Posada, D. and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14:817-818.

Purpura, K., D. Trachina, E. Kaplan, and R. M. Shapely. 1990. Light adaptation in the primate retina: analysis of changes in gain and dynamics of monkey retinal ganglion cells. *Vis. Neurosci.* 4(1):75-93.

Rens-Domiano, S. and H. E. Hamm. 1995. Structure and functional relationships of heterotrimeric G-proteins. *FASEB J*. 9:1059-1066.

Rock, B., L. Xin, and J. N. Wilcox. 1997. Two Gq class G proteins are expressed in human keratinocytes. *J. Inves. Derm*. 109:645-649.

Sandhya, K. and C. Vemuri. 1997. Regulation of cellular signals by G-proteins. *J. Biosci.* 22:375-397.

Sarwar, M. M., J. Sontag, L. Hoang, S. Brennerand T. M. Wilkie. 1996. G protein alpha subunit multigene family in the japanese puffer fish *Fugu rubripes*: PCR from a compact vertebrate genome. *Gen. Res.* 6:1207-1215.

Schmitt E. A. and J. E. Dowling. 1999. Early retinal development in the zebrafish, *Danio rerio*: Light and electron microscopic analyses. *The Journal of Comparative Neurology* 404:515-536.

Shah, B. H. and R. E. Hausman. 1996. Developmental expression of G-proteins in embryonic chick retina. *International. J. Dev. Neurosci.* 14(51):92-92.

Shapira, M., M. Gafni, and Y. Sarne. 1998. Independence of, and interactions between cannabinoid and opioid signal transduction pathways in N18TG2 cells. *Brain Res.* 525:225-231.

Simon, M. I., M. P. Strathmann, and N. Gautam. 1991. Diversity of G proteins in signal transduction. *Science* 252:802-808.

Sprang, S. R. 1997. G protein mechanisms: Insights from structural analysis. *Annu. Rev. Biochem.* 66:639-678.

Strathmann, M. P. and M. I. Simon. 1991. G alpha 12 and G alpha 13 subunits define a fourth class of G protein alpha subunits. *Proc. Natl. Acad. Sci. U .S. A.* 88(13):5582-5586.

Suzuki, T., K. Narita, K. Yoshihara, K. Nagai, and Y. Kito. 1995. Phosphatidyl inositol-phospholipase C in squid photoreceptor membrane is activated by stable metarhodopsin via GTP-binding protein Gq. *Vision Res.* 35:1011–1017.

Swofford, D. L. 1998. PAUP*. Phylogenetic Analysis Using Parsimony. Version 4. Sinauer Associates, Sutherland, Massachusetts.

Takasaki, J., T. Saito, M. Taniguchi, T. Kawasaki, Y. Moritani, K. Hayashi, and M. Kobori. 2004. A Novel G{alpha}q/11-selective Inhibitor. *J. Biol. Chem.* 279:47438-47445.

Tatusova, T. A. and T. L. Madden. 1999. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. *FEMS. Microbiol. Lett.* 177(1):187-188.

Taylor, S. J., H. Z. Chae, S. G. Rhee, and J. H. Exton. 1991. Activation of the β1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. *Nature* 350:516-518.

Terakita, A., H. Takahama, S. Tamotsu, T. Suzuki, T. Hariyama, and Y. Tsukahara. 1996. Light-modulated subcellular localization of the alpha-subunit of GTP-binding protein Gq in crayfish photoreceptors. *Visual Neurosci.* 13:539–547.

Thompson, J. D., D. G Higgins, and T. J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nuclei. acids Res.* 22:4673-4680.

Ushikubi, F., K. Nakamura, and S. Narumiya. 1994. Functional reconstitution of platelet thromboxane A2 receptors with Gq and Gi2 in phospholipid vesicles. *Mol. Pharmacol.* 46:808-816.

Venkatakrishnan, G. and J. H. Exton. 1996. Identification of determinants in the alpha-subunit of G(q) required for phospholipase C activation. *J. Biol. Chem.* 271:5066-5072.

Vivas, I. M. and B. D. Drujan. 1980. Certain aspects of acetylcholine mechanism in teleost retina. *Neurochem. Res.* 5(8):817-829.

Walker J. E., M. Saraste, M. J. Runswick and N. J. Gay. 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* 1:945–951.

Wettschureck, N. and S. Offermanns. 2005. Mammalian G proteins and their cell type specific functions. *Physiol. Rev*. 85:1159-204.

Wilkie, T. M., D. J. Gilbert, A. S. Olsen, X. Chen., T. T. Amatruda, J. R. Korenberg, B. J. Trask. P. Jong, R. R. Reed, M. I. Simon, N. A. Jenkins, and N. G. Copeland. 1992. Evolution of the mammalian G-protein alpha multigene family. *Nature Genetics* 1:85-93.

Wilkie, T. M. and S. Yokoyama. 1994. Evolution of G protein alpha multigene family. *Soc. Gen. Physiol. Ser*. 49:249-270.

Wu, D., A. Katz, C. H. Lee, and M. I. Simon. 1992. Activation of phospholipase C by α1-adrenergic receptors is mediated by the alpha subunits of the Gq class. *J. Biol. Chem*. 267:25798-25802.

Yajima, Y., K. Uchino, H. Ito, and S. Kawashima. 1997. Mastoparan-stimulated prolactin secretion in rat pituitary GH3 cells involves activation of Gq/11 proteins. *Endocrinology* 138:1949-1958.

Yokoyama, S. and W. T. Starmer. 1992. Phylogeny and evolutionary rates of G protein α subunit genes. *J. Mol. Evol*. 35:230-238.

Zaunreiter, M., R. Brandstätter, and Goldschmid, A. 1998a. Evidence for an endogenous clock in the retina of rainbow trout: I. Retinomotor movements, dopamine and melatonin. *Neuroreport* 9:1205–1209.

Zaunreiter, M., R. Brandstätter, and A. Goldschmid. 1998b. Evidence for an endogenous clock in the retina of rainbow trout: II. Circadian rythmicity of serotonin metabolism. *Neuroreport* 9:1475–1479.

Zinn, K. M. and M. F. Marmor. 1979. *The retinal pigment epithelium*. 521 Harvard University Press, Cambridge, MA.

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