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A Study of Biliverdin, Serum Albumin and Hemoglobin in the Scincid Lizard, Prasinohaema

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A Study of Biliverdin, Serum Albumin and Hemoglobin in the Scincid Lizard, *Prasinohaema*

By

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Abstract

Lizards of the genus *Prasinohaema* are the only vertebrates with high concentrations of the toxic bile pigment biliverdin in the blood. Elevated levels of bile pigments in the blood cause jaundice in all vertebrates except for these unusual lizards. I explored several aspects of this strange physiology, including its potential medical application – the condition of green jaundice in humans is caused by biliverdin (the related molecule bilirubin causes clinical jaundice). Species of *Prasinohaema* have a very large concentration of biliverdin in their blood, yet they show no ill effects due to this, presenting only a green coloration of the blood, bones and mucosal tissues (Austin and Jessing, 1994). I have explored some of the aspects of biliverdin as well as aspects of molecules that interact with it, and I will detail these studies in this thesis, as well as my lab work pursuing the genetic sequence of hemoglobin from *Prasinohaema*.

Chapter 1 will detail the biochemistry of biliverdin and bilirubin, focusing on the metabolism of these chemicals starting with the breakdown of heme. It will also detail some of the clinical conditions that occur in humans during jaundice, and briefly discuss research focusing on treatment for this condition.

Chapter 2 analyzes the relationship between serum albumin and biliverdin/bilirubin. Serum albumin is the principle carrier of these molecules in the bloodstream, and as such is worth investigation (for an additional carrier of biliverdin, see appendix 2). The free bilirubin in the blood (not bound to serum albumin) is a major cause of jaundice in humans.

Chapter 3 discusses some of the properties of hemoglobin, including its structure and function in different species of vertebrates. Also I will describe in this chapter my lab work dedicated towards sequencing the Alpha-D hemoglobin gene in *Prasinohaema*.

This research underscores the importance of the biochemical pathway producing biliverdin, as well as the importance of serum albumin and hemoglobin. These are fascinating subjects, and I look forward to seeing how research will advance in the coming years in regard to these topics, especially as they apply to the green-blooded lizards of *Prasinohaema*.

Introduction

The purpose of this research project was to sequence a hemoglobin gene in *Prasinohaema*, so that I could study one of the possible explanations for the high concentration of biliverdin in the lizard's tissues. Biliverdin is also produced in humans, so there is a medical application to this research. It is possible that the hemoglobin in *Prasinohaema* is different than the hemoglobin in red-blooded lizards. There could be several reasons behind this if it is true. First, the hemoglobin may have undergone an adaptation over time because of changes in its environment due to the large concentration of biliverdin. This would be a result of the biliverdin abundance rather than a cause of it. Another possibility is that a difference in hemoglobin structure led to a difference in the way the enzymes degraded it, causing the abnormal accumulation of biliverdin. Perhaps more biliverdin is produced in the lizards due to a structural difference in the hemoglobin molecule itself. It would present differently to the degradation enzymes, and this could cause an increase in the rate at which they degraded it, producing a larger baseline biliverdin concentration. Or, biliverdin levels might increase if the structural change provided the lizards with hemoglobin of a shorter lifespan. My hypothesis was that a mutation in the hemoglobin gene led to structural change in the hemoglobin of *Prasinohaema,* which in turn led to an increase in the frequency of its degradation by enzymes. My attempt to sequence the Alpha-D hemoglobin of *Prasinohaema* was a search for evidence to support or contradict this hypothesis.

Chapter 1: Jaundice: A Medical Problem Rooted in Biochemistry

In the space of one month in summer 2012, an outbreak of jaundice in the town of Maharashtra, India, affected over 3500 people and was fatal to 21. Schools, colleges and restaurants were shut down for eight days in the wake of the crisis, and the two local hospitals were completely filled with patients. The local health department suspects that the outbreak started from the polluted Panchganga River that provides much of the town's drinking water. A widespread infection from this water may have produced the jaundice in the population (CNN IBN, June 20, 2012).

Jaundice is still a modern health problem, as this report shows. It is more common in underdeveloped countries, and it also tends to affect newborns (neonatal jaundice) more often than it does adults. Here I will explore the biochemical basis of jaundice, and describe how these biochemical aspects medically affect the jaundiced person.

Jaundice is a yellow coloring of the skin, mucus membranes, or the whites of the eyes. Besides the pigmentation, symptoms of jaundice include weakness, headache, nausea, and dull pain in the liver region (Jaundice, PubMed NCBI). This condition occurs when bilirubin, a chemical produced when red blood cells are broken down at the end of their life cycle, collects in the tissues of the body.

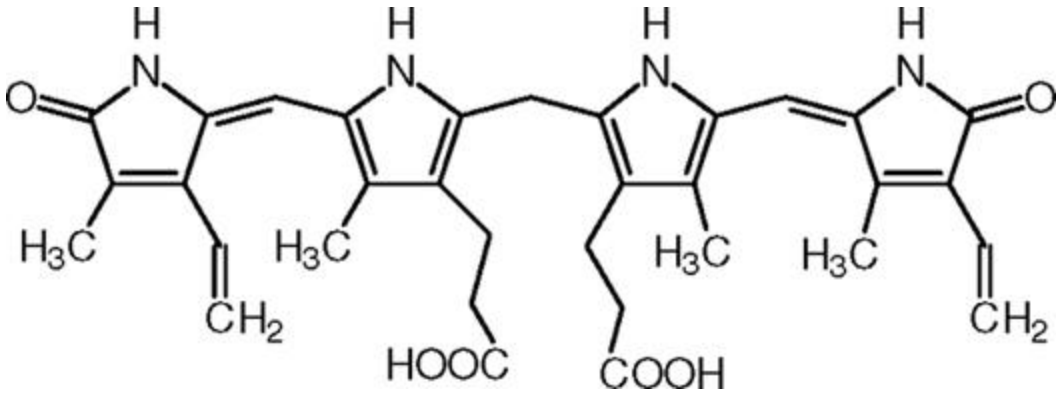

Figure 1 – the chemical structure of bilirubin – Oxford Dictionary of Biochemistry, 2014

Bilirubin (see figure 1) is a bile pigment – normally it becomes a part of bile after being metabolized by the liver (Gray, 1958). The accumulation or "clumping" of bilirubin that occurs in jaundice only happens at much higher concentrations than normal (normal range 0.3-1.0 mg/dL). Jaundice results from very large amounts of bilirubin in the body (>3.0 mg/dL) (Sargent and Clayton, *Adult Jaundice*). The primary organ that is responsible for controlling bilirubin levels is the liver.

Jaundice is often a symptom of a liver disease or of some damage to that organ. In the case of the outbreak in India in 2012, a hepatitis virus that causes damage to the liver may have been the cause of the widespread jaundice (CNN, 2012). If the damaged liver cannot properly metabolize the bilirubin, the bloodstream levels of this chemical will increase, and jaundice will occur as the pigment accumulates. The presence of bilirubin in the body starts with the breakdown of red blood cells.

Red blood cells (erythrocytes) are the most common source of bilirubin. Bilirubin is the end product of the heme degradation pathway, and red blood cells are the chief source of heme in the body (from hemoglobin). Erythrocytes have a normal life span of around 110 days,

after which they are broken down and recycled (Sargent and Clayton, 2011). The rest of the bilirubin in the body is derived from heme proteins not from red blood cells (such as myoglobin) (Sargent and Clayton, 2011).

Heme degradation involves two main chemical processes. The first process occurs in the macrophages of the spleen, liver and bone marrow. Heme oxygenase catalyzes a reaction that removes the iron and carbon monoxide from the heme molecule. This reaction produces biliverdin, which is the pigment responsible for the green color sometimes seen in bruises. The carbon monoxide is transported to the lungs and exhaled from there, while the iron is recycled (Sargent and Clayton, 2011).

In the second process, biliverdin is converted to bilirubin. The enzyme that catalyzes this reaction is biliverdin reductase, a cytosolic enzyme (Greenburg, 2002). At this point, the bilirubin is insoluble (unconjugated) due to internal hydrogen bonds that it can form, exposing its non-polar regions and making the molecule hydrophobic. To be excreted it needs to be converted into a water-soluble (conjugated) form (see figure 2). The unconjugated bilirubin at this point is transported in the blood plasma to the liver (via the albumin in the blood), where the hepatocytes metabolize it (Sargent and Clayton, 2011).

Researchers think that organic anion transporting polypeptide (OATP) helps extract the bilirubin from the blood plasma, and transport it to the hepatocyte. Then the unconjugated bilirubin is sent to the endoplasmic reticulum of the hepatocyte, after which it is transported across the cytosol, and transformed into the conjugated (polar) form. An enzyme, uridine diphosphate glucuronoryl transferase, helps this reaction occur. In the reaction, glucuronic acid

(figure 3) conjugates to the propionic acid side chains of the bilirubin molecule (Sargent and Clayton, 2011).

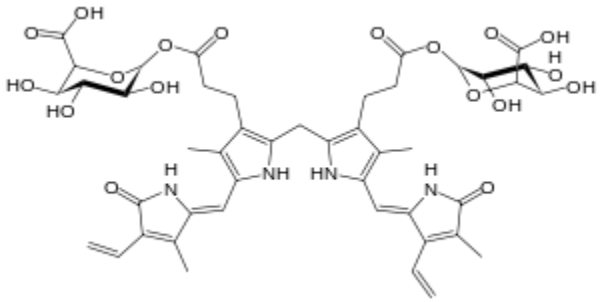

Figure 2
Conjugated bilirubin, (Lengyel, G)

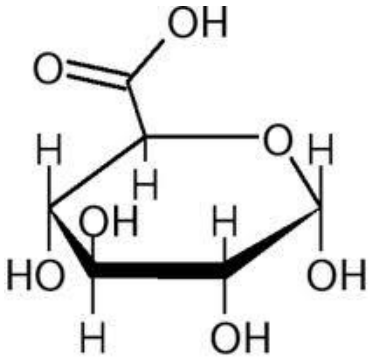

Figure 3
Glucuronic acid, Sigma Aldrich (USA), sigmaaldrich.com

As mentioned before, unconjugated bilirubin hydrogen bonds to itself, becoming hydrophobic. Conjugating the bilirubin with glucuronic acid allows the molecule to stay polar. Also, the toxic effects associated with bilirubin occur when the bilirubin is in its unconjugated form. So conjugation of bilirubin aids the body in several ways (Sargent and Clayton 2011).

After this reaction, the now water-soluble bilirubin is sent by an ATP export pump into the bile canaliculi, where it combines with other molecules to form bile. The bile is sent to the duodenum, where the bilirubin is converted to stercobilin, most of which is excreted in the feces (With, 1968). So through this pathway, most of the body's bilirubin is eliminated and not

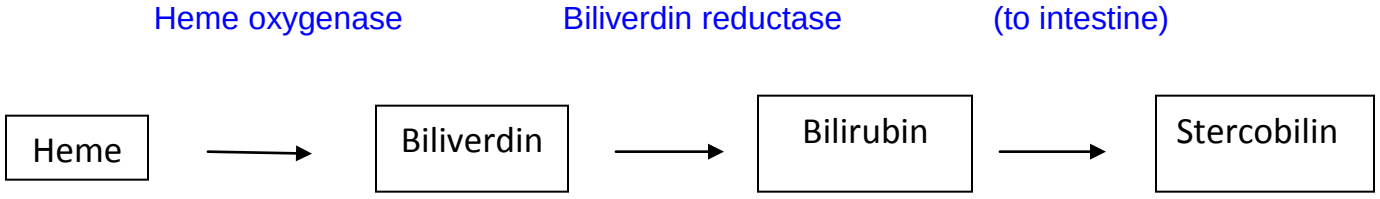

Figure 4: Overall heme degradation pathway

allowed back into the bloodstream (see figure 4).

Jaundice occurs when either the liver can't conjugate the bilirubin quickly enough to keep up with the large amounts being produced (known as pre-hepatic jaundice), when the bilirubin is not properly excreted into the bile canaliculi (hepatic jaundice), or when bile is not secreted properly into the duodenum (post-hepatic jaundice) (Gray, 1958). Any of these cases can lead to excess bilirubin in the body, which can deposit in tissues, leading to the symptoms of jaundice.

Bilirubin's structure is important to its function - bilirubin is the most commonly encountered natural linear tetrapyrrole (Bulmer et al, 2008). A tetrapyrrole is a molecule with 4 pyrrole groups, which are 5-membered rings containing a nitrogen atom, as seen in figure 1. The natural stereoisomer of bilirubin is the Z,Z form (where the larger priority groups are on the same side for both double bonds), although this form can be converted to the E,Z form when exposed to light (the E,Z form is more water-soluble than the Z,Z form, as it has a more open structure). Research actually indicates that the molecule is not linear in its natural form, but two of the pyrrole groups are perpendicular to the other two, forming a large "L" shape (Bonnet, et al, 1976). As mentioned before, the water-soluble conjugated form has glucuronic acid chains attached to it, which allows it to be secreted and joined to bile molecules.

Excessive bilirubin in the bloodstream (hyperbilirubinemia) can cause brain damage if left unresolved for extended periods of time. Nearly all the bilirubin in the blood is bound to albumin (before the bilirubin reaches the liver), so the small fraction of free bilirubin is responsible for jaundice when it occurs in humans. Bilirubin causes a rapid increase in cellular apoptosis (programmed cell death), by increasing the polarity of the lipid layer in nerve cells,

especially those in the grey matter of the central nervous system. It also increases the fluidity of the lipids and injures the lipids through oxidation (Rodrigues, et al 2002). To summarize, bilirubin interacts with lipid membranes causing death of nerve cells (Yin and Waterai, 2007).

Although bilirubin can cause problems such as nerve damage in addition to causing the yellow color of jaundice, there is evidence that suggests that it can be helpful in small quantities (McDonagh, 2001). For example, bilirubin may destroy reactive oxygen species such as peroxyl radicals that cause harm to cells in the body (Van Hoydonka et al, 2001). Though small amounts of bilirubin in the body may be beneficial, larger amounts put a person at risk for nerve damage.

The potential that free bilirubin may cause neurological damage is one of the main concerns for newborns who show signs of jaundice. Jaundice is most common in humans soon after birth, as various statistics say that from fifty to sixty percent of newborns have some form of jaundice (Iacob et al, 2011). Normally the jaundice occurs within twenty-four hours of birth, and spontaneously disappears within a week or two. It is a common problem in infants due to the time it takes for the baby to adjust to its own metabolism. The mother's body discards the bilirubin for the baby through the placenta before birth, so after birth many newborns need a week or so to be able to fully metabolize all the bilirubin that needs to come through the liver (Iacob et al 2011).

Iacob et al (2011) studied neonatal jaundice in newborns at a Romanian hospital during the years 2008 and 2009. In their study, 35 newborns at the hospital had some form of jaundice. Out of these infants, the majority needed only phototherapy to be restored to normal

skin pigmentation. None of the 35 infants in the study died. Neonatal jaundice, although common, is neither deadly nor does it require aggressive treatment.

Phototherapy consists of shining a blue light on a newborn in an effort to break down the bilirubin in the skin (Bakalar, 2011). The light energy causes the bilirubin to isomerize into a water-soluble form, which the body can then excrete. As the predominate natural isomer is the Z,Z form, light changes the molecule to either the E,Z form or some other polar form which the body can excrete. So the phototherapy takes advantage of the light energy to change the chemical structure of the bilirubin, allowing its elimination. This treatment is painless and relatively efficient – usually the phototherapy needs to continue for only a day or two (Khumer and Bhomick 2010).

As jaundice is most often classified as a symptom rather than a disease, in adults the treatment of jaundice depends on the underlying condition. These treatments could range from antibiotics to treat an infection, or removal of a tumor on the liver, or even to a liver transplant in cases where the liver is severely damaged. In any case, in adults, jaundice is a result of a more serious problem than that of an infant who is working out his own metabolism pathway for bilirubin. Another treatment idea was mentioned by Lavin et al (1985). They suggested that a small blood filter composed of bilirubin oxidase (which degrades bilirubin) could be placed in the blood to filter out the bilirubin. In their study, 90 percent of the bilirubin in the blood was degraded on a single pass through the filter (Lavin et al, 1985).

This project relates to jaundice via the scicnid lizard *Prasinohaema.* The lizards in this genus have green blood; the pigment causing this coloration is biliverdin, the same pigment that leads to jaundice in humans when converted to bilirubin. However, these lizards are viable

and healthy, though they have an overwhelmingly large concentration of biliverdin in their blood plasma – approximately 40 times higher than humans with green jaundice (the version of jaundice caused by excess biliverdin) (Austin and Jessing, 1994).

Biliverdin may not be quite as toxic as bilirubin, at least as studied in tissue cell cultures. However it still seems that these lizards should show some side effect from the overwhelming quantity of biliverdin concentrated in their bodies. Several hypotheses have been proposed that suggest reasons for this amount of biliverdin. These include UV protection, as the biliverdin could possibly protect the lizard from harmful rays, as well as thermoregulation – perhaps the biliverdin keeps the lizard's body temperature stable. The biliverdin may also make them distasteful - predators then would avoid attacking the lizard to eat it (Austin and Jessing 1994). Or these lizards could be mimicking a distasteful lizard. However these are all hypotheses; none has yet been rigorously tested.

To conclude, jaundice is caused by an accumulation of bilirubin. Bilirubin is a unique structure derived from heme, much of which comes from red blood cells. The degradation pathway of heme produces biliverdin, then bilirubin, which is normally disposed of in the liver through bile and conversion to stercobilin. When this pathway is not followed successfully, for example if the liver is damaged and cannot keep up with the load of bilirubin it is receiving, the bilirubin levels can rise in the bloodstream. This molecule then travels throughout the body and aggregates, giving skin, mucous membranes and the eyes a yellow coloring. Jaundice is fundamentally a biochemical problem – bilirubin is the direct cause of the condition.

Chapter 2: Bile Pigments and Serum Albumin

Summary: Serum albumin has an interesting relationship to biliverdin and bilirubin. I studied these two bile pigments, their source, and their transport in the bloodstream in different vertebrates. These pigments are produced through the heme degradation pathway, which has differences between mammals (the pathway ends at bilirubin), and other vertebrates – birds, reptiles, amphibians and fish (the pathway stops at biliverdin). Serum albumin transports these pigments in the bloodstream in a very specific molecular orientation. Lysine and arginine residues are critical for the binding of bilirubin in a hydrophobic pocket of serum albumin, and a conformational change of the serum albumin molecule occurs as the binding happens (Gonchorova and Urbanova 2008).

Serum albumin is a multifunctional transport molecule – it binds and delivers several other ligands besides bile pigments, such as fatty acids, small ions and drugs. Serum albumin is highly conserved throughout vertebrates – its size, amino acid sequence and function as a transporter and osmotic regulator do not change much from humans and other mammals to birds, reptiles and fish. And this serum albumin molecule has unique properties. It binds a broad spectrum of substances, yet it still binds with incredible specificity. It can bring various molecules to specific sites, and does so with efficiency and accuracy (Nicholson et al, 2000).

In general, bile pigments are noted mostly for excess buildup of bilirubin in tissues, called jaundice (see ch 1). The buildup of bile pigments that causes jaundice comes from bilirubin being free and unbound from serum albumin, due to excess bilirubin in the body, more than the serum albumin can handle. I want to explore the way in which these pigments bind

serum albumin. First, I will present a phylogenic perspective on heme degradation, exploring differences in this pathway between mammals, birds, other reptiles, and fishes. I then want to do the same with serum albumin, exploring its structure and properties in mammals, as well as in other vertebrates. I finally want to examine the specific mode in which serum albumin binds biliverdin and bilirubin.

Other vertebrate heme degradation pathways The heme degradation pathway discussed in chapter 1 applies to mammals with only slight variations between species. However, in birds, reptiles and amphibians, biliverdin is the end product of heme degradation. So the metabolic pathway in these animals does not continue past the heme oxygenase reaction, shortening the overall pathway by a step. The heme oxygenase enzyme in fact is common to many organisms, including some bacteria (Unno et al 2007). That aspect of this pathway seems to be a universal trademark.

In fishes, bile pigments can occur in the scales, skin, bones and other organs. This occurs in healthy fish, as it does in *Prasinohaema,* and is non-pathological. It is suspected that in these animals the pigments have some specific function, as opposed to being metabolic by-products. However thus far this is not supported with any evidence (With, 1968).

Serum albumins in mammals: Serum albumin, the carrier of bilirubin and biliverdin is the most abundant plasma protein in humans, as well as in many other animals. It is a member of a super-family that includes vitamin D-binding protein, alpha albumin and alpha-fetoprotein, all of which are also carrier molecules (See appendix 1 for nomenclature details). Serum albumin's amino acid sequence has been discovered in humans. It contains 585 amino acids,

with a molecular weight of 66.5 kDa. It has an abundance of charged residues, and few tryptophan or methionine residues (Nicholson et al 2000).

The shape of human serum albumin has been described as a heart (see figure 5), 80A by 30A. The mature molecule is arranged in a series of alpha-helices, folded and held by 17 disulfide bridges. It has 3 main domains (from which the heart shape comes) each made of 2 subdomains. The molecule is very flexible, and changes shape readily with environmental changes and with ligand binding. But it is still very resilient,

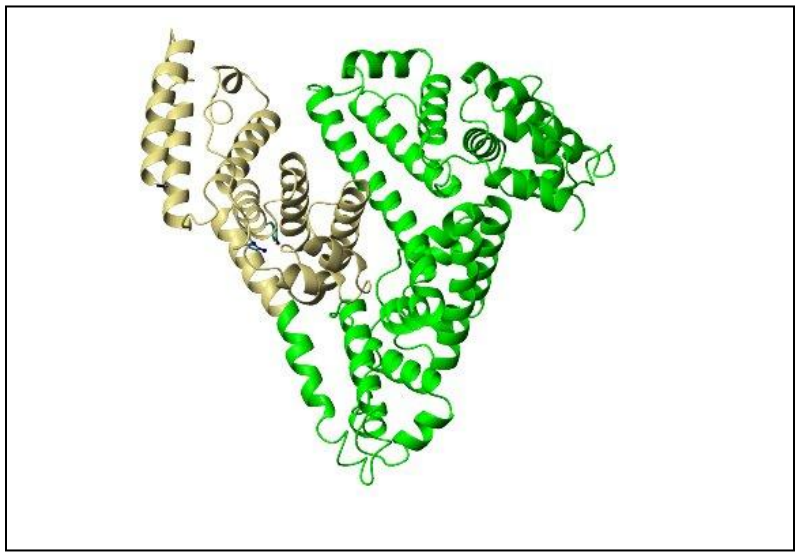

Figure 5: Structure of Serum Albumin (hawaii.edu)

regaining shape easily due to the disulfide bridges. The albumin molecule is only denatured under dramatic conditions, such as extreme temperature or pH (Nicholson et al 2000).

In a healthy adult human, there is about 250-300 grams of albumin in the body. Some of it is lost to tissue or other regions of the body, and the liver replenishes the supply through

synthesis of new albumin. Approximately 12-25 grams of albumin is secreted (and lost) each day in a healthy adult. Albumin is a protein, so the rate of its synthesis depends on the abundance of necessary amino acids, presence of mRNA for translation, and ribosomal machinery for the synthesis of the molecule (Nicholson et al 2000).

Most organs in the body break down albumin. Muscle and skin tissue break down the largest percentage of albumin rather than the liver, although approximately 10% of the breakdown does occur in hepatic tissue. The mechanism of breakdown includes uptake into endocytotic vesicles, which fuse with lysosomes. The lysosomes degrade the albumin into amino acids, which can circulate back into tissues to build more proteins. Thus albumin can serve as a reservoir of amino acids for the body (Nicholson et al 2000).

One of albumin's most important properties is its binding capability. It is a carrier of a broad spectrum of materials which are important to health by virtue of either their necessity or toxicity. Since the albumin molecule is flexible, some toxic substances can be buried in the molecule and nearly hidden from the bloodstream. The molecules that are most strongly bound are medium sized hydrophobic organic anions, such as fatty acids, bilirubin and haematin. Molecules that are either smaller or more hydrophilic can also be bound, but they bind with less affinity than these optimum substances. Albumin does have a distinct negative charge, but oddly the charge of the binding ligand does not correlate strongly with the binding affinity of the molecule (Nicholson et al 2000).

Some of the other substances that albumin binds are ions such as copper and zinc, eicosanoids as well as other fatty acids, and vitamin D. Albumin also carries steroids – these have a lower affinity for the albumin, but since albumin is the main carrier in the bloodstream,

due to sheer numbers a significant portion of the steroids will be carried by albumin. Many drugs given to humans for various reasons also bind to serum albumin. They bind to a wide variety of sites on the molecule, chiefly on subdomains IIa and IIIa (Nicholson et al 2000).

The above discussion serves to illustrate the multifarious functions of serum albumin. Nearly 100 genetic variants of the serum albumin molecule have been discovered (chiefly through electrophoretic mobility studies). However, only one variant has been known to change the function of the molecule; that is the variant that causes dysalbunemiaemic hyperthyroidism. This condition is due to the albumin's increased affinity for thyroxine due to the genetic variation. The other variants have no problem performing the functions of a normal albumin molecule (Nicholson et al 2000).

Serum albumin variations in other vertebrates: The tuatara (*Sphenodon punctatus*) is unique among terrestrial vertebrates in that it does not seem to possess a 60 – 75 kDa serum albumin. Its albumin is 130 kDa, but this protein seems to function just as the smaller albumins do in other vertebrates. Some of the characteristic genetic sequences are conserved with other animals too, making this size of albumin very unique and puzzling. A possibility for the origin of this molecule is that there was a duplication of the albumin gene at some point in the tuatara's ancestry, leaving the very similar function to other species' albumin, but a size that is twice as large (Brown et al 1995). This variant of albumin may give the animal a certain advantage of some sort, but this is unknown and doubtful, as no variant of human albumin has yet been seen to produce any advantage or disadvantage.

The red-eared slider turtle (*Trachemys scripta elegans*) contains two variants of the serum albumin molecule. These two molecules have similar amino acid sequences, and very

similar properties. This is a case of alloalbunemia, a condition which again seems to have little significance on vertebrate life, as the two albumin molecules each function correctly with no obvious issues. One of the albumins, (Alb-1) has a histidine content twice as high as the other albumin (Alb-2). And Alb-2 has twice as many isoleucine residues than Alb-1. A feature that the two variants in this turtle have in common with chicken and tuatara is an acidic residue at position +3 that is not present in mammal albumin (Brown et al 1997). This difference means that most reptiles lack a strong metal binding site that mammals possess.

As a third vertebrate example, the common carp (*Cyprinus carpio*) has no albumin-like protein similar to human serum albumin. The experiment done by De Smet et al (1998) explored fatty acids, and how they were bound in the bloodstream. They found that free fatty acids were bound to high density lipoprotein (HDL), and they suggested that this HDL can act as an alternate carrier for fatty acids in the absence of serum albumin. Serum albumins or albumin-like proteins have been found in the rainbow trout, and the Atlantic salmon, so albumin is know in some species of fish (De Smet et al 1998).

Circulatory transport of molecules and osmotic balance occur in fishes as well as land-dwelling vertebrates, and these functions of albumin are performed in the plasma of fishes by different types of molecules. The rainbow trout does contain a serum albumin molecule, but it does not carry all of the free fatty acid molecules in the bloodstream like human serum albumin does. Other fatty acid molecules are carried by lipoprotein as occurs in the carp. Thus the rainbow trout uses a combination of serum albumin and HDL to transport fatty acids (De Smet et al 1998).

As these examples show, many species of vertebrates have a similarly functioning serum albumin. Albumin itself, though it has a broad range of functions, does not vary much between different species – it is relatively conserved. It is an important molecule, especially as a carrier. One of the important groups of molecules that it carries is the bile pigments.

Serum albumin binding biliverdin and bilirubin: Serum albumin binds bilirubin and biliverdin in the bloodstream. Although serum albumin has broad binding capacity, it does have specific sites for binding bile pigments. Both biliverdin and bilirubin form non-covalent complexes with serum albumin, at a ratio of one bile pigment molecule per serum albumin molecule. Non-conjugated free bilirubin is poorly soluble in water, as discussed above, so it is conjugated with glucuronic acid residues in the liver to ensure excretion in the bile. Before conjugation, serum albumin plays a critical role in reducing the toxicity of bilirubin by increasing its solubility. Binding bilirubin allows it to travel in the bloodstream (Alyoff et al 1980). I want to discuss a couple of projects that examine the conformation of the bound pigment, what amino acids bind it, and whether the reduction of biliverdin occurs while the pigment is bound to serum albumin.

The first question that arises is whether the reaction reducing biliverdin to bilirubin occurs while the pigments are bound to serum albumin, or whether free biliverdin is converted to bilirubin. Since the binding constant of biliverdin to bovine serum albumin is on the order of 10^6 1/M, (high affinity) and similarly the binding constant of bilirubin is around 10^8, Claret et al (1995) considers the conversion of free biliverdin to bilirubin unlikely. This means that the reduction of biliverdin to bilirubin takes place while the bile pigments are complexed with their carrier, serum albumin. The experiment by Claret et al (1995) used electroreduction to change

biliverdin to bilirubin, and they discovered only slightly higher rates for reduction of free pigment verses reduction of the bound pigment. So this indicates a strong possibility that the in vivo reduction of biliverdin to bilirubin occurs as the pigments are bound by serum albumin (Claret et al 1995).

Light absorption data (Blauer and Wagniere 1975) indicates that there is a conformational change when serum albumin binds a bile pigment. This change indicates a specific change in the chirality of the bile pigment, again as seen through the absorption data. Biliverdin complexed with serum albumin is skewed in a left-handed conformation. Bilirubin is also left-handed, with a specific angle between the 2nd and 3rd nitrogen-containing rings – this angle may vary slightly in different conditions (Khan and Tayyab 2001). One model of bilirubin binding, the two half-domain model, has each dipyrrole unit of the pigment binding to separate subdomains of albumin. This is supported by the absorption spectrum of bilirubin, and shows why the angle between the two halves of the molecule is crucial for the molecule's positioning.

There is a broad range of possibilities for the angle mentioned above, but the idea of some specific angle does support the idea of a highly specific mode of binding of the molecules as they are carried by serum albumin, most likely in a relatively tight helical conformation for bilirubin. There is no free rotation in biliverdin unlike bilirubin, due to the double bond between the 2nd and 3rd dipyrromethanes. Thus the three-dimensional structures of biliverdin and bilirubin differ greatly; biliverdin adopts a helical, lock-washer shape, while bilirubin has a folded ridge-tile molecular geometry (Gonchorova and Urbanova 2008).

The binding site on both human and bovine serum albumin that displays high affinity for bilirubin and biliverdin is subdomain IIA (amino acid positions 190-300). There is extremely high

homology between the II domains in bovine and human serum albumin. This highly homologous site binds small molecules with carboxyl groups as well as bile pigments (Gonchorova and Urbanova 2008). Bilirubin binds to albumin at or near loop 4 in subdomain IIA – this binding is stabilized by non-covalent forces such as salt linkages, hydrogen bonds, van der Walls interactions and hydrophobic interactions. The most important bonds are the salt linkages between NH2 groups of interior lysine and arginine residues, and the carboxyl groups of bilirubin. It is unusual for charged residues to be in the protein interior – more normally they are on the exterior of the protein (Gonchorova and Urbanova 2008). This abnormality is crucial however, as it enables bilirubin to be carried in the interior of the albumin molecule.

If you take away the charged residues (Lys and Arg), then the hydrophobic interactions and Van der Waals forces are sufficient to hold the bilirubin molecule in the albumin pocket, but the interaction occurs at a much reduced affinity. Taking away the lysines also changed the geometry of the binding site, which again reduced the affinity for bilirubin. So lysine positioning is crucial for binding the pigment (Gonchorova and Urbanova 2008). (Khan et al (2001) performed an experiment by succinilating and acetylating lysine residues – blocking the lysines – which reduced albumin's affinity for bilirubin).

The way in which pigments bind serum albumin depends on the location of the Lys and Arg residues in the binding site. Lys 199 is involved in the formation of the bile pigment-serum albumin complex; it lies in a hydrophobic pocket, with its side chain pointing towards the entrance channel of the binding crevice. Lys 195, Arg 218 and Arg 222 (also positively charged residues) are nearby in the hydrophobic pocket, and may also form critical salt linkages with carboxylate groups of bilirubin and biliverdin. There are 16 hydrophobic amino acids that form

the pocket (slightly different in bovine vs human serum albumin; approximately 3 residue changes, but all are still hydrophobic). One interesting difference between bovine and human serum albumins is that the biliverdin binding site is closer to the surface of the molecule in cows, while it's more buried in humans. Sheep serum albumin is similarly homologous, with the same charged residues, and 2 hydrophobic residue swaps. Guinea pig, horse, rabbit and other mammals have the same binding site pattern – charged residues (Lys or Arg) within a hydrophobic pocket as well (Khan and Tayyab 2001).

In addition to binding bile pigments, the internal lysine residues of serum albumin stabilize the tertiary structure of the serum albumin molecule. So the salt linkages of internal lysine residues are important for binding the bile pigments, and they provide stability to the three-dimensional structure of the bound pigment (Khan and Tayyab 2001). They actually change the conformation of the pigment, in the way discussed above, as well as stabilize the serum albumin molecule.

Conclusions: The heme degradation pathway produces biliverdin (chiefly from red blood cells), which is then converted to bilirubin and excreted in mammals. Biliverdin is the end of the heme degradation pathway in other vertebrates; the pathway stops for these organisms after the heme oxygenase reaction. Serum albumin is a multi-functional molecule that carries an array of pigments, ions, drugs and fatty acids, among other molecules. It is highly conserved among different species of vertebrates, both in structure and function. Finally, serum albumin binds biliverdin and bilirubin with high specificity. More is known about the mechanism of bilirubin's binding, especially the importance of charged residues in serum albumin interacting with the carboxyl groups of bilirubin. Yet for both molecules there is a conformational change

of the serum albumin when the pigments are bound, indicating specific interactions with amino acids at a specific location in the albumin molecule.

Chapter 3: Alpha-D hemoglobin in *Prasinohaema*

Introduction: Hemoglobin is a critical component of the vertebrate respiratory system, and an absolute necessity for the survival of many organisms. This molecule transports oxygen from an organism's oxygen-intake system to respiring tissues in nearly every vertebrate species, as well as in many invertebrate species (Snyder 1999). Some hemoglobin can be found in tissues, but it is most commonly found as an integral part of the red blood cell (Snyder 1999). In addition to this critical function, as I mentioned earlier, the breakdown of heme (part of hemoglobin) is the crucial first step in the biliverdin/bilirubin metabolism pathway. So for my exploration of *Prasinohaema*, hemoglobin is an important molecule to consider. My goal was to sequence a hemoglobin gene in the green-blooded *Prasinohaema* to see whether it differs from the hemoglobin of red-blooded lizards.

Hemoglobin's structure in vertebrates is known to consist of four polypeptide chains, with each chain containing a heme group. This heme group is non-organic, containing an iron molecule. One oxygen molecule can bind to each heme group, thus a single hemoglobin molecule can carry up to four oxygen molecules at one time. Each erythrocyte possesses many hemoglobins at one time, thus one erythrocyte can transport many oxygen molecules to the body tissues (Sherwood, 2013).

Each of the four subunits of hemoglobin can bind an oxygen molecule as mentioned above; in humans, two of these subunits are alpha-hemoglobin, and the other two are beta-hemoglobin. Asakura and Lau (1978) performed an experiment to study the oxygen-binding capabilities of the two hemoglobin chains. They discovered that there is no binding preference

between the alpha and beta chains of hemoglobin. However, they did notice that in the presence of organic phosphate, for some reason oxygen binds preferentially to the alpha-chains of hemoglobin. They also noticed that the binding of an oxygen molecule to one heme molecule affects the conformation of the other subunits. This can change their affinity for oxygen as well, enabling cooperative binding of the other oxygen molecules – these next oxygen molecules will bind more easily after the first oxygen has been bound (Asakura and Lau, 1978).

In many vertebrates, including humans, there are variants to the alpha and beta hemoglobin molecules. For example, in chickens (*Gallus gallus*) there are two types of alpha hemoglobin, alpha-A and alpha-D (Richards and Wells, 1980). In other vertebrates additional types of alpha and beta globin genes are expressed, including variation of beta chains in addition to alpha chain variants. The green anole lizard, *Anolis carolinensis,* has two types of beta hemoglobin, Beta-1 and Beta-2, and two types of alpha hemoglobin, Alpha-D and Alpha-A. *Anolis* lacks an Alpha-E gene, perhaps due to a deletion, which makes it unique among tetrapod vertebrates (Storz, 2010).

Materials and Methods: I wanted to explore the hemoglobin genes present in *Prasinohaema*, as hemoglobin's may be an underlying cause for the large biliverdin concentration in this genus (since it is at the beginning of the heme degradation pathway). Or at least it might have an evolved protein sequence change as a result of transporting oxygen in the circulatory system with such a high concentration of biliverdin (see chapter 1 for the heme degradation pathway). So I began by collecting and aligning several of the common types of alpha and beta hemoglobin sequences using the online program Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo). I researched hemoglobin from some turtle species, as well

as from chicken (*Gallus gallus*), and the lizard *Anolis carolinensis*. All of these species had their sequences readily published and available on NCBI's Genbank tool (ncbi.nlm.nih.gov/genbank). Several of the DNA sequences yielded reasonably close alignments to each other, and I decided to use an Anolis/chicken alignment while pursuing the sequence of the *Prasinohaema* gene. The alpha-D gene was the simplest hemoglobin gene to pursue out of the options, so it made a logical starting point for a sequencing project.

Alignment: The alpha-D hemoglobin sequence alignment between *Gallus gallus* and *Anolis carolinenesis* included introns and exons of their respective alpha-D hemoglobin genes, as well as 200 base pairs upstream and downstream for each sequence. The length of the total alignment was nearly 1700 base pairs. This alpha-D gene has 3 exons. The first had a length of about 100 base pairs (bp), the second had a length of about 200 bp, while the third had a length of about 150 bp. The exons were much more conserved across the two species than the more lengthy introns were.

Primers: As the next step in the sequencing process, I designed PCR primers with which to amplify the *Prasinohaema* Alpha-D hemoglobin gene. These primers should work if the gene region for which the primers were developed is conserved from chicken to *Anolis carolinenesis* to *Prasinohaema*. The Primer3 tool from the University of Massachusetts medical school served as a good tool to design the primers (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). I created 6 different pairs of primers, programming Primer3 to pick from the areas on the alignment that seemed most conserved to me (where the two sequences had the most base pairs in common – marked by an asterisk in Clustal). Two forward primers were upstream of Exon 1, two were in the midst of

Exon 1, and two were in the midst of Exon 2. Two of the reverse primers were also in the midst of Exon 2, two were in Exon 3, and two were downstream of Exon 3. My plan was to amplify the entire gene if possible, or otherwise do it in two pieces with the break coming in Exon 2 (as the primer's location suggests – see Appendix 3 for the alignment, Appendix 4 for the list of primers).

Extractions: I performed standard DNA salt extractions on several samples of lizard tissue from the LSU Museum of Natural Science. The two species of *Prasinohaema* used were *Prasinohaema flavipes* (Accession numbers: CCA 1658, CCA 1666, CCA 1646, CCA 1658, CCA 1671) and *Prasinohaema prehensicauda* (CCA 1687, CCA 1674, CCA 1674, CCA 1675, CCA 1684). In addition, I extracted DNA from the closely related red-blooded lizards *Lipinia noctua* (CCA 5529, CCA 5566) and *Lobulia elegans* (CCA 1639, CCA 1789, CCA 1677, CCA 1678) to make a comparison of the Alpha-D hemoglobin between the red and green-blooded lizards. A difference between the two hemoglobin molecules could be responsible for the notable differences in pigmentation between the two varieties of lizard – perhaps they wouldn't degrade in the same manner if there are differences in structure.

PCRs: After extracting DNA, I ran several gradient PCR reactions to explore which of my primers sets would be the most successful. A gradient PCR uses a range of annealing temperatures for the PCR reaction – this allows the experimenter to determine the optimal temperature for the primer set used. So I ran this reaction to amplify the alpha-D hemoglobin gene for potential sequencing.

Results: I used three of my best DNA extractions (For *P. flavipes, L. noctua,* and *L. elegans)* for PCR's using two of my primer sets, and had some partial success, managing to

amplify some unconfirmed segments of the Alpha-D hemoglobin gene in these three lizards. (From the alignment that I used, I estimated the Alpha-D hemoglobin gene in *Prasinohaema* to be about 1250 base pairs long)

The primers that I picked should serve to help amplify most of the hemoglobin gene for the lizard species. One primer began upstream of exon 1, and ended in exon 2. So this segment would include all of exon 1, as well as all of the first intron. The second primer set began in exon 2, and ended in exon 3. The amplification product from this reaction would include much of exons 2 and 3, as well as all of the intron between these two segments. I had more success with this second amplification, as the agarose gel I ran containing the PCR products showed DNA in a couple of lanes of approximately 400 base pairs. This corresponded to the target DNA sequence I was looking for. This has yet to be analyzed by sequencing at the present moment.

Discussion: The looming end of my undergraduate studies reduced the time available for the full exploration I had intended, thus I was not able to attempt all of my primer combinations. However it is still possible that some of them may have shown some promise. Further research will hopefully continue along this path – the hemoglobin sequence data from these green-blooded lizards may prove useful in determining the reason for their distinctive coloration. Even a single amino acid change in the Alpha-D hemoglobin molecule (at the beginning of the heme degradation pathway) may have consequences related to the large build-up of biliverdin in the blood and tissues of the *Prasinohaema* genus. A large difference between the *Prasinohaema* hemoglobin gene and the *Anolis* or *Gallus* genes may explain the fact that the designed primers did not work instantly or ideally. And perhaps this difference

could even be at the root of the vast difference in pigmentation between *Prasinohaema* and other taxa on this planet.

Appendix 1: Confusion over the nomenclature lies in the use of the term “albumin”. Albumin is commonly used to describe proteins similar to serum albumin that exist in plants, animal tissues, eggs and milk. It is also used as a name for any water soluble protein. To make matters worse, serum albumin is sometimes simply called albumin. There is a group of related proteins that is called the albumin super-family. This super-family includes serum albumin, vitamin D-binding protein, alpha-fetoprotein, and alpha-albumin. These four proteins are different structurally and are produced by different genes, but they retain some similarities including an exon splicing pattern and a pattern of disulfide bridges (Richard and Wells 2012).

These four proteins are thought to be evolutionarily related. The genes for these three proteins lie near each other in the human genome. Alpha-fetoprotein is only seen in infants, and is thought to be the fetal version of serum albumin, necessary in development.

Appendix 2: The lipocalins are a family of proteins in the body that transport small hydrophobic molecules, such as steroids, lipeds and retinols. The unifying characteristic of this family is its 3-dimensional structure, which includes a beta-barrel (essentially a beta-sheet rolled up). The molecules to be transported are bound inside this structure, at a specific binding site (Miyamoto, et al 2010). The lipocalins have only limited sequence homology; the "kernel lipocalins" (the most conserved relative to each other) do have 3 conserved sequences of amino acids. There are 17 kernel lipocalins, each composed of 160 – 185 amino acids. Prostaglandin-D synthase, one of these kernel lipocalins, is known to complex with biliverdin, and act as a transporter for that molecule (Flower 1996).

Appendix 3: Alignment:

CLUSTAL O(1.2.0) multiple sequence alignment

Anolis Carolinensis lizard and *Gallus gallus* (chicken)

Hemoglobin Alpha-D sequence, with 200 bp above and below coding sequence

53% overall similarity

Exon 1
Exon 2
Exon 3
Introns

```
                5'                                                    AHupF2
Anolis          ----------------------GAAGGGAGGGGCCAAGGAGGAAAGGCGCAGACCCCCCCC
Chicken         CCCCCTCAGGGCCCCCTCTGTGGGCATCGGGGCCCCTGAGGCACCGC---------CGCTC
                5'                      *.. . .******.. ****.*. **        * * *

Anolis          CCTCCTT-TGGCTCTGTCCTGCTCCAAA----CCCAGAGGGAGATAAGAGGGGTGGGCCC
chicken         CCTGCTCTCAGCATTGCACAGCCACGGCCCCTCCGTGCGGATAAGATAAGGCCGGGGCGG
                *** **   .**: ** .*:** .*...    ** :*.**.:.* *:.***   ****

                          AHupF1
Anolis          CGGAGACGGGAGCTATAAAGGCAGCTGCCTCTTTGAGCCGTGGCTCCGAGCCCCACTGCC
Chicken         GTGTACAGGGAGCTATAAGAGGTCGGCCCTGCAGGCT-----CCTCCATCACACATTGCC
                  *:...***********..* :    ***  : *.       ****.: .*.** ****
                                                          AHinF3
Anolis          ACTGATCCCGAAAGAGAGTCATCCTTGAGAGGAGAAACCAGACAAGATGGTGCTGACCGC
Chicken         ACCAGCCACC------AG----------CCCGCCCCACCAGCTGCCACCATGCTGACTGC
                ** .. *.*       **           . *. ..*****. .. *  .******* **
                                                                  AHinF4
Anolis          TGAGGACCGCAAACTGCTGACAGCCATTTGGGGGAAGGTGTCTGGGCACCTGGATGAGAT
Chicken         CGAGGACAAGAAGCTCATCCAGCAGGCCTGGGAGAAGGCCGCTTCCCACCAGGAGGAGTT
                 ******.. **.** .* ... . .  ****.*****   **   ****:*** ***:*

Anolis          CGGCGGGGATGCTCTCAACAGGTGAGCAGATGGGGAGAGAGGGAAGCATGGGGTGTTGGG
Chicken         TGGAGCTGAGGCTCTGACTAGGTGCGAGCCAGGCCCAGGGG---CACCTGGCGGGGTGGG
                 **.*  ** ***** *. *****.*.. .:**  ...*.*   ..*.*** * * ****

Anolis          GACTAGCCCAGAGGGGGAGTAAAGGAGAGAATTCCAGGAGAGCAATAAAACA-AGCCTTT
Chicken         AATTGGG---GAGTTGGAGCCAAGGGGTGCGGGCCAGGTCCTGGAGTGTTGGGGTGTGTG
                .* *.*    ***  **** .****.*:*..  *****: .  .* :.:: . .    *

Anolis          TATTATCATAGCTGATGATAAGGCAAAACAGCCATAGGTACCCACATTTTGTGGGTCCGT
Chicken         GGTTGGGGCAGGGGGAACTGCGGGGCAGCAGAGCTGACCAGCCTC---------------
                 .**.  . **  *.:..*..** ..*.***. .*..  * **:*

Anolis          ACCATCCAAATGGCCGTTGCACCATGTGCGTAGCAAACAAAGGATTTTATATCTCAGCCT
Chicken         ------------------------------------------------------------


Anolis          TATCTAGAATTGACCAATGGCTGAGAGTCTTTCCCACCTTCCACACCTACTTTGGCACAA
Chicken         ------------------------------------------------------------
```

```
Anolis      GTGATATCAATGACCCAACTTGTTTACTCTTTCTTCTCCCTCTCAAACTTCTTGAAGAAA
Chicken     ------------------------------------------------------------

Anolis      GGCACCAGCTCACAGGAAGGGAGGGGCACAGGCCCTGTGTTGCACATACTACTTTGATTC
Chicken     ------------------------------------------------------------

Anolis      ATACAAAGTGCTTATATTATCTATATAAAGGTATATATGATACATGCAGAGGCAAAGCTA
Chicken     ------------------------------------------------------------

Anolis      TATAAACAATAGTCTACTATTTTCTGGCTTATGGCCCCTACAAGGGAGGGGAGAGAAATG
Chicken     ------------------------------------------------------------

Anolis      AAGAAGAGCCCGGGAGCCTGTCTTCTTCCAGCCCTCTCTGAGGCATCCCTCTTTGTTCCT
Chicken     ------------------------------------------------------------

Anolis      TCTTTCCTTTCGCTTCTGGCTCCCTTTTCCTTTGATGCAGGATGTTCATGTGTTTCCCAT
Chicken     ------------------------------CTTCCGGCAGGATGTTCACCACCTATCCCC
                                           ** . ************  :  *: **.
                   AHmidF5 & AHmidR6
Anolis      CGACTAAGACCTACTTCCCGCACTTCGACCTGAGCCCGGGCTCCAAGGACATCCAAGTGC
Chicken     AGACCAAGACCTACTTCCCCCACTTCGACCTTTCGCCTGGCTCTGACCAGGTCCGTGGCC
            .*** ************** *********** :  ** ***** .*  * .***.:*  *

Anolis      ATGGCCAGAAGGTGGCCAGAGCCCTGGACATGGCCCTCCACCACCTGGACAACGTCCGTG
Chicken     ATGGCAAGAAGGTGTTGGGTGCCCTGGGCAACGCCGTGAAGAACGTGGACAACCTCAGCC
            *****.********   .*:*******.**: *** * .* .** ******** **.*
                          AHmidF6                    AHmidR5
Anolis      GCACCCTGGCTGACCTCAGCGACCTCCACGCCTACAACTTGCGAGTCGACCCCGTCAACT
Chicken     AGGCCATGGCTGAGCTGAGCAACCTGCATGCCTACAACCTGCGTGTTGACCCCGTCAATT
            . .**.******* ** ***.**** ** ********* ****:** ********** *

Anolis      TCAAGGCAAGCCCCGCTGGGAGGGAAAGGCCGAGGGGGGAAAG-----------------
Chicken     TCAAGGCAAGCAAAGGCTGGGGTCTGCGGGTCCTGGGGTCCTCAGGTCAGGGGATCTTGG
            ***********...*   **.*  :..**   . **** ..:

Anolis      ------------------------------------------------------------
Chicken     GGTTACAGGATCCTGGAGGTCTTGGGGTCAGGGGGTCCTAGAGTAAAGGGGTCCTGAGGT

Anolis      ------------------------------------------------------------
Chicken     CAGGGTGTGCTGGGTTCCTGGGAGCTGGGATCCTAGGGATCAGGAGCCAGAGGCCAGGGG

Anolis      --------------------------GCAAGGAGGGAGTTCTCCTCTCCCGCTGACCCCT
Chicken     TGGTACCAGGGCCAGAATGGGGGACAGAATGGGACTGAGAGTCCTCCATCCAGAGCAAGG
                                      *.*:**..  .. : ***** . * . ..*..

Anolis      GACACCCCCTTTTTCTCTTTCTGCAGCTGCTGATCAAGTGCTTCCACGTGGTTCTGGCCA
Chicken     GTACTGAGCCTCTGTTTGCCTTGCAGCTGTTGTCGCAGTGCATCCAGGTGGTGCTGGCTG
            *:..  . * * *  *     ******** **:  .*****:**** ***** ***** .
```

```
                                AHinR4
Anolis      CTCACCTGCGCGGAGAGTACACCTGCTTGGCCCACCGGGCTGTGGACAAGTTCTTATTCG
Chicken     TACACATGGGCAAAGACTACACCCCTGAAGTGCATGCTGCCTTCGACAAGTTCCTGTCTG
             :***.** **..*** ******    :.*  **    **  * ********* *.*  *
                                AHinR3
Anolis      CGGTGGCCGAGATCCTGGGCGAGAAGTACAGATGAGCAGACAAAGGAGAAGACCCCCCTA
Chicken     CCGTGTCTGCTGTGCTGGCTGAGAAGTACAGATAAGCCACCATCTACAACTTCAAGTCTT
            * *** * *. .* ****  *************.***...**:. ...*. :*..  **:
                             AHdownR1
Anolis      TCTTTTCTTCAGAGACTCCCCTTCTTCTTCATCTAG---AATTTTCCCT--TCCCAGCAT
Chicken     ------CAATAAAGACACCATTGCTGCAGCACTGTGTCCATGTGTGCTGGGGCTGGGGAC
                  *:: *.****:**. * ** *: **   :*   *: * * *     *  .* *

Anolis      TCGCAATAAAAACAAGGCCTTTGCCGCCACCTGCCTAATGCTGTCTGAGTCTGGCTTTTT
Chicken     AGGGCATAGGGGTCCAGGGTGGGCTGGGGCACACTCAATGCTTCCCCACACCCCTTGGGA
            : * .***.... ...*  *  ** *  .*. .*  ******  *  * :*    *   :
                                          AHdownR2
Anolis      TGA-AGGAGAGAGG-------GGTCCTTTGTTTCAGGGGGAATCCCCATTTCACACAAGA
Chicken     GGGAGGGAGAAAGGAAGCTGCTAGCAGGTCTGGCATGGGGCCTGCCTGGTTAAAC-GTGA
             *. .*****.***        . *.  * *  ** ****..* ** . **.*.. .:**

Anolis      AGCCCCTC  3′
Chicken     TC------  3′
            :
```

KEY

An * (asterisk) indicates positions which have a single, fully conserved residue.
A : (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.
A . (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.

Appendix 4: Primers

Primers outside all exons

1

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER AHupF1	94	19	60.18	57.89	4.00	2.00	CCGGAGACGGGAGCTATAA
RT PRIMER AHdownR1	1401	22	59.99	50.00	5.00	1.00	TGAAGAAGAAGGGGAGTCTCTG

SEQUENCE SIZE: 1545
INCLUDED REGION SIZE: 1545

PRODUCT SIZE: 1308, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 0.00
TARGETS (start, len)*: 115,1250
EXCLUDED REGIONS (start, len)*: 1,90 1410,135

2

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER AHupF2	15	18	60.50	61.11	4.00	1.00	AGGAGGAAAGGCGCAGAC
RT PRIMER AHdownR2	1520	20	60.66	45.00	3.00	0.00	ATTCCCCCTGAAACAAAGGA

SEQUENCE SIZE: 1545
INCLUDED REGION SIZE: 1545

PRODUCT SIZE: 1506, PAIR ANY COMPL: 6.00, PAIR 3' COMPL: 0.00
TARGETS (start, len)*: 55,1440
EXCLUDED REGIONS (start, len)*: 1532,13

Primers within exons

3

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER AHinF3	191	20	59.26	50.00	4.00	2.00	ACCAGACAAGATGGTGCTGA
RT PRIMER AHinR3	1341	20	59.31	55.00	4.00	2.00	CTGTACTTCTCGCCCAGGAT

SEQUENCE SIZE: 1545
INCLUDED REGION SIZE: 1545

PRODUCT SIZE: 1151, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00
TARGETS (start, len)*: 240,1050
EXCLUDED REGIONS (start, len)*: 1,190 1355,185

4

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER AHinF4	257	18	60.00	55.56	3.00	2.00	TGGGCACCTGGATGAGAT
RT PRIMER AHinR4	1281	20	59.87	60.00	4.00	2.00	GCCAAGCAGGTGTACTCTCC

SEQUENCE SIZE: 1545
INCLUDED REGION SIZE: 1545

PRODUCT SIZE: 1025, PAIR ANY COMPL: 6.00, PAIR 3' COMPL: 3.00
TARGETS (start, len)*: 290,950
EXCLUDED REGIONS (start, len)*: 1,240 1315,215

5.
```
OLIGO            start  len      tm      gc%   any   3'  seq
LEFT PRIMER AHmidF5 29   21    59.77    52.38  3.00  1.00 AAGACCTACTTCCCGCACTTC
RIGHT PRIM AHmidR5 194   20    58.52    55.00  6.00  3.00 GGTCGACTCGCAAGTTGTAG
SEQUENCE SIZE: 210
INCLUDED REGION SIZE: 210

PRODUCT SIZE: 166, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00
EXCLUDED REGIONS (start, len)*: 1,26
```

6.
```
FORWARD AHmidF6: TGGCTGACCTCAGCGACCTCCA            (same locus as R5)
  REVERSE AHmidR6: GAAGTGCGGGAAGTAGGTCTT     forward is downstream of reverse
```

Primer Glossary:

start (Start Position)
The position of the 5' base of the primer. For a Left Primer or Hyb Oligo this position is the position of the leftmost base. For a Right primer it is the position of the***rightmost*** base.

len (Oligo Length)
The length of the primer or oligo.

tm (Melting Temperature)
The melting temperature of the primer or oligo.

gc%
The percent of G or C bases in the primer or oligo.

any (Self Complementarity)
The self-complementarity score of the oligo or primer (taken as a measure of its tendency to anneal to itself or form secondary structure).

3' (Self Complementarity)
The 3' self-complementarity of the primer or oligo (taken as a measure of its tendency to form a primer-dimer with itself).

rep (Mispriming or Mishyb Library Similarity)
The similarity to the specified Mispriming or Mishyb library.

seq (Primer Sequence, 5'->3')
The sequence of the selected primer or oligo, always 5'->3' so the right primer is on the opposite strand from the one supplied in the source input. (The right

primer sequence is the sequence you would want synthesized in a primer order.)

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