Duplication and diversification of insulin genes in ray-finned fish

ABSTRACT

Insulin is a key hormone for the regulation of metabolism in vertebrates. Insulin is produced by pancreatic islet cells in response to elevated glucose levels and leads to the uptake of glucose by tissues such as liver and adipose tissue to store energy. Insulin also has additional functions in regulating development. Previous work has shown that the proglucagon gene, which encodes hormones counter regulating insulin, is duplicated in teleost fish, and that the peptide hormones encoded by these genes have diversified in function. I sought to determine whether similar processes have occurred to insulin genes in these species. Searches of fish genomes revealed an unexpected diversity of insulin genes. A triplication of the insulin gene occurred at the origin of teleost fish, however one of these three genes, insc, has been lost in most teleost fish lineages. The two other insulin genes, insa and insb, have been retained but show differing levels of selective constraint suggesting that they might have diversified in function. Intriguingly, a duplicate copy of the insa gene, which I named insab, is found in many fish. The coding sequence encoded by insab genes is under weak selective constraint, with its predicted protein sequences losing their potential to be processed into a two-peptide hormone. However, these sequences have retained perfectly conserved cystine residues, suggesting that they maintain insulin’s three-dimensional structure and therefore might modulate the processing and secretion of insulin produced by the other genes.

INTRODUCTION

Fish have been important contributors to our understanding of human biology and disease (Lieschke & Currie, 2007; MacRae & Peterson, 2015), especially in endocrinology (Conlon, 2000a). In vertebrates, insulin is a hormone produced by the beta-cells of pancreatic islets in response to increased levels of blood glucose, which induces the uptake of glucose by tissues such as liver and adipose tissue for storage, and thus is a key regulator of glucose metabolism (Röder et al., 2016). Deficiencies in insulin production and/or signaling leads to diabetes in humans and other animals (Röder et al., 2016; Weiss, 2009). Many aspects of insulin function have been learned from extensive studies in diverse species of fish (Caruso & Sheridan, 2011; Conlon, 2000b; Polakof et al., 2012). Fish have also been explored in the development of potential treatments for diabetes. Xenotransplantation of fish islets, using both wild-type and humanized insulin, has been considered due to the relative ease of isolation of islets from pancreatic tissue in fish (e.g., tilapia, Oreochromis niloticus) compared to mammalian sources (Wright et al., 2014). Insulin, however, in addition to its role in the regulation of metabolism, also has other roles in vertebrate biology, including some in development (Chan & Steiner, 2000; Hernández-Sánchez et al., 2006).

Insulin is a polypeptide hormone composed of two peptides, A- and B-chains of about 20 and 30 amino acids, respectively, which are held together by disulfide bridges (Conlon, 2001; Steiner et al., 1985, 2009; Weiss, 2009). Insulin sequences have been characterized, mostly at the protein level, in a large number of vertebrate and fish species (Caruso & Sheridan, 2011; Conlon, 2000b, 2001). Typically, vertebrate species have a single copy of the insulin gene in their genomes (Nishi & Nanjo, 2011; Steiner et al., 1985), however, multiple insulin genes have been characterized in some species, such as
rodents (Long et al., 2013; Soares et al., 1985; Wentworth et al., 1986) and some fish (Caruso et al., 2008; Caruso & Sheridan, 2011, 2014; Irwin, 2004). Within fish, it appears that only one of these insulin genes, which produces the hormone that regulates blood glucose levels, is expressed in the pancreas (Caruso & Sheridan, 2011; Irwin, 2004), with the exception of the multiple insulin genes produced by the very recent genome duplication in salmonid fish (Caruso et al., 2008; Caruso & Sheridan, 2014). While the origin of the two insulin genes found in some fish (e.g., zebrafish) was shown to be early in teleost fish evolution, whether this ins2 gene has been retained in the genomes of diverse fish is unclear (Caruso & Sheridan, 2011; Irwin, 2004). The fish ins2 gene has been poorly characterized. The initial identification of this gene in zebrafish (Danio rerio) and takifugu (Takifugu rubripes) only showed expression in the embryo of zebrafish (Irwin, 2004). A subsequent study of the expression of the two insulin genes in zebrafish suggested that ins2 potentially has a function as a growth and neurotrophic factor during development (Papasani et al., 2006). More recently, studies of the tilapia (O. niloticus) ins2 gene showed that it had negligible expression in the pancreas, thus likely is not a major contributor to the regulation of glucose metabolism and would not need to be silenced to allow the xenotransplantation of tilapia islets as a treatment for diabetes in humans (Hrytsenko et al., 2016).

Changes in the function of insulin can be paralleled by changes in the function of glucagon, the hormone that counters the action of insulin (Seino et al., 1988). Recently it has been shown that duplicated proglucagon genes are widespread in teleost fish, and that the functions of the proglucagon-derived peptides encoded by these genes have diversified (Irwin & Mojsov, 2018). Here I have taken advantage of the large number of fish genomes that have been characterized in the past few years (Ravi & Venkatesh, 2018) to determine the distribution of insulin genes in the genomes of ray-finned fish. Analysis of these sequences allows us to begin to understand the origin of these genes, including the measurement of the selective forces acting upon them, which might provide evidence for differences in function. Surprisingly, I found a greater diversity in the numbers of insulin genes than expected, with the identification of a third insulin paralog, as well as lineage-specific duplicates of some of these paralogs. My analyses suggest that all three fish insulin paralogs encode functional protein products.

**MATERIALS AND METHODS**

**Data collection**

Genome sequences databases maintained by the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/genome/gdw/) and Ensembl (http://www.ensembl.org) were searched in March/April 2018, for sequences that were predicted to encode proteins similar to proinsulin. Initial searches used the tBLASTn algorithm (Gertz et al., 2006) using previously characterized zebrafish (Danio rerio) proinsulin protein sequences (Irwin, 2004; Milewski et al., 1988) as queries. Subsequent tBLASTn, BLASTn, and BLASTp searches used our putative proinsulin sequences as queries. Genome sequences of 55 fish (52 ray-finned (Superclass Actinopterygii), 2 cartilaginous (Superclass Chondrichthyes), and one lobe-finned (Class Sarcopterygii)) were examined, with 52 of these contained in the NCBI Genome Data Viewer, and three (Atlantic cod, Gadus morhua; three-spined stickleback, Gasterosteus aculeatus; and the spotted green pufferfish, Tetraodon nigroviridis) only in the Ensembl database (9 were in both NCBI and Ensembl). A list of species with genomes used here, and their phylogenetic relationships, is presented in Figure 1. All sequences with E-scores below 0.001 were examined. As an initial step to assess homology and orthology, reciprocal BLASTp searches of the zebrafish proteome were conducted to examine the similarity of the putative proinsulin sequences. Sequences that were not more obviously similar to insulin-like growth factor-I (igf1) or insulin-like growth factor-II (igf2) were used for the following analyses. Here I suggest a standardized set of names for fish ins genes, based on the analysis described below, with insa, insb, and insc representing the three ins paralogs that originated early in teleost fish evolution, and insaa and insab representing duplicates of the insa gene shared by multiple species. Arabic numerals are used for recent lineage-specific duplicates and do not indicate necessarily indicate orthology.

**Phylogenetic analysis**

Prior to phylogenetic analysis, proinsulin (ins) coding sequences were aligned using the MAFFT algorithm (Katoh et al., 2001) as implemented at the Guidance2 server (http://guidance.tau.ac.il/ver2/; Penn et al., 2010). Phylogenetic relationships were established using both maximum likelihood and Bayesian methods. PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/; Guindon et al., 2010) was used to generate maximum likelihood trees, with 500 bootstrap replications, where the Smart Model Selection (SMS) (Lefort et al., 2017) option was used to identify the best fitting evolutionary model. Bayesian trees were constructed using MrBayes 3.2.6 (Huelsenbeck et al., 2001; Ronquist et al., 2012), with 5 000 000 generations and four simultaneous Metropolis-coupled Monte Carlo Markov chains sampled every 100 generations under the same evolutionary model used for maximum likelihood. The first 25% of the trees were discarded as burn-in with the remaining samples used to generate consensus trees. Insulin coding sequences from cartilaginous (Callorhinichus milli (Elephant shark) and Rhincodon typus (Whale shark)) and lobe-finned fish (Latimeria chalumnae (Coelacanth)) were used as outgroups to root the trees for all insulin coding sequences. Subsequent analyses, using subsets of the sequence data, were conducted with similar approaches, and used sequences identified in our initial analyses as outgroups.

**Evolutionary analysis**

Genes adjacent to insulin genes in fish genomes were identified by examining genomic contigs in the NCBI and Ensembl databases using methods that I have previously used (Irwin, 2002, 2012; Irwin & Mojsov, 2018). Briefly, the identity and orientation of genes neighboring insulin genes were identified.
The orthology of the genes adjacent to insulin genes was assessed (or confirmed if annotated) using BLASTp searches of the zebrafish and human proteomes (Kuraku & Meyer, 2012).

Figure 1 Diversity and evolution of fish insulin genes

Summary of the evolution of insulin genes in ray finned fish. Phylogenetic relationships of species with genome sequences is based on Betancur-R et al. (2017), with modifications in an area that is not confidently resolved to minimize the number of gene duplications or deletions. Some phylogenetic relationships are not resolved. Branches are not proportional to evolutionary time. Classification of fish is shown to the right. Solid diamonds indicate genome duplications. The rates of nonsynonymous (dNS) and synonymous substitutions (dS) ratios. dNS/dS rate ratios were obtained from analyses using RELAX (Wertheim et al., 2014) as implemented on the Datamonkey Adaptive Evolution server (http://datamonkey.org; Weaver et al., 2018), which also tested for intensification or relaxation of the levels of selection on tested lineages.
Evidence for positive selection on branches of the phylogenetic tree was tested using aBSREL (Smith et al., 2015) on the Datamonkey Adaptive Evolution server (Weaver et al., 2018).

**Prediction of protein processing sites and alignment of peptide sequences**

Signal peptidase cleavage sites in the proinsulin protein sequences were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/; Petersen et al., 2011). I used two prediction programs to identify potential prohormone protease processing sites in the proinsulin sequences, NeuroPred (http://neuroproteomics.scs.illinois.edu/neuropred.htm; Southey et al., 2006) and ProP 1.0 (http://www.cbs.dtu.dk/services/ProP/; Duckert et al., 2004), with “general PC prediction” selected. To visualize the conservation of the processing sites and insulin peptide sequences, the proinsulin protein sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Ots/msa/clustalo/; Sievers et al., 2011). Consensus proinsulin A- and B-chain peptide sequences were generated from the alignments using WebLogo 3 (http://weblogo.threeplusone.com/; Crooks et al., 2004).

**RESULTS**

**Numbers of insulin genes in fish genomes**

Searches of genome databases maintained by NCBI and Ensembl resulted in the identification of 168 insulin genes from the genomes of 55 species examined, with 141 of these predicted to be intact full-length coding sequences (Figure 1 and Supplementary Table S1). While only single copy insulin genes were found in the genomes of cartilaginous and lobe-finned fish, multiple genes were found in the genomes of most ray-finned fish. Within ray-finned fish, the number of identified insulin genes ranged from 1 (Sclerophages fromorus) to 8 (Sinocyclocheilus anshuiensis). As expected, fish species that experienced recent genome duplications, i.e., carp (Cyprinus carpio; Xu et al., 2014), members of the genus Sinocyclocheilus (Yang et al., 2016), and salmonids (Onchorhynchus kisutch, O. mykiss, Salmo salar, and Salvelinus alpinus; Lien et al., 2016) have the largest numbers of insulin genes. Phylogenetic analysis (see below) suggested the presence of three insulin paralogs in teleost fish: insa, insb, and insc. Of the 162 insulin genes identified in the 51 species of teleost fish (Infraclass Teleostei), 92 were classified as insa (which includes insaa and insab genes), 57 as insb, and 13 as insc, with 11, 12, and 3 of the sequences found to be incomplete, respectively, for these three paralogs (Figure 1 and Supplementary Table S1). All 51 species of teleost fish possessed at least one insa gene, with 45 having insb genes, and 10 with insc genes (Figure 1 and Supplementary Table S1). The 6 species of teleost fish where I failed to identify insb genes are distributed across the accepted phylogeny of fish (Figure 1; Betancur-R et al., 2017; Near et al., 2012), suggesting they were lost independently several times (or are missing from these genome assemblies). In contrast, almost all of the species lacking insc genes share a common ancestor (Figure 1, clade Euteleostei), suggesting a single gene loss event explains the absence of insc genes in most teleost fish. The coding sequences of the insulin (ins) genes used in our analysis described below are presented in Supplementary Figure S1.

**Phylogeny of fish insulin genes**

To better understand the origin and evolution of the multiple insulin (ins) genes found in the genomes of ray-finned fish, their phylogenetic relationships, rooted using ins gene sequences from cartilaginous and lobe-finned fish, were inferred using maximum likelihood (Figure 2) and Bayesian (Supplementary Figure S2) methods. Both analyses generated similar phylogenies with independent duplications of the insulin gene on the spotted gar (Lepisosteus oculatus; infraorder Holostei) and teleost (infraorder Teleostei) fish lineages. While duplicated insa and insb genes were previously identified in teleost fish (Caruso & Sheridan, 2014; Hrytsenko et al., 2016; Irwin, 2004), here I find that these species have a third insulin paralog, which I named insc. The species relationships within each paralog of insulin genes, given its limited power at resolving species relationships, is in general agreement with the accepted phylogeny of teleost fish (Betancur-R et al., 2017; Near et al., 2012). This observation suggests that the triplication of the insulin gene occurred in an early teleost. The relationships among the insa, insb, and insc paralogs is poorly resolved in our phylogenetic analyses, with the maximum likelihood analysis suggesting a closer relationship between insa and insb, while the Bayesian analysis suggesting that insb and insc are most closely related.

Most teleost species that possess insb and insc genes have a single copy of each gene, except those species that have experienced additional genome duplications (i.e., Cyprinus carpio, salmonids (O. mykiss, O. kisutch, Salmo salar, and Salvelinus alpinus), and the genera Sinocyclocheilus)). In contrast, a large number (30 of 51) of teleosts have multiple insa genes (Figure 1 and Supplementary Table S1). While some of these appear to be due to lineage-specific genome duplications (i.e., Cyprinus carpio, salmonids, and the genera Sinocyclocheilus) or single-gene duplications (e.g., Labrus bergylta and Larimichthys crocea), many are due to a duplication event that occurred on an early Percomorpha lineage (Figures 1, 2, and Supplementary Figure S2). The genomes of many Percomorpha fish contain two (or more) insa genes that form monophyletic clades that are largely in accord with the accepted species phylogeny (Betancur-R et al., 2017; Near et al., 2012). This observation suggests that a duplication of the insa gene, generating insaa and insab genes, occurred in an early lineage of Percomorpha fish (see Figure 1). In species that are inferred to be descendants of this Percomorpha-specific ins gene duplication, all but Nothobranchiuis furzeri possess an insaa gene, however, a larger number of species, such as tilapia (Oreochromis niloticus), some cichlids (e.g., Pundamilia nyererei and Maylandia zebra), and species of the genus Poecilia do not have an insab gene (Figures 1, 2 and Supplementary Figure S2 and Table S1). This suggests that the insab gene was lost in parallel in a number of species of Percomorpha (see Figure 1).
Phylogenetic relationship of insulin coding sequences was inferred using maximum likelihood with the best fitting evolutionary model GTR+G+I (GTR with gamma distribution and invariant sites) and bootstrapped with 500 replications. A similar phylogeny was generated using Bayesian methods (Supplementary Figure S2). Percent bootstrap support for lineages are shown. The tree in Newick format, with all branch lengths and support values, is presented in Supplementary Figure S3. The phylogeny was rooted with sequences from cartilaginous (Callorhinus milii and Rhincodon typus) and lobe-finned (Latimeria chalumnae) fish. Full species names are listed in Figure 1. insa, insb, and insc indicate the three clades of insulin genes found in teleost fish.
Duplications of fish insulin genes

To aid in the assessment of the orthology of insulin genes, and to gain insight into the gene duplication events, I identified genes flanking the insulin genes in the genomic sequences of 55 species of fish (Supplementary Table S2, examples given in Figure 3). A genome duplication occurred in the ancestor of teleost fish, the teleost-specific genome duplication (Glasauer & Neuhauss, 2014; Meyer & Van de Peer, 2005). Our phylogenetic analyses (Figure 2 and Supplementary S2) suggests that the three insulin paralogs found in teleost fish (insa, insb, and insc) originated on this lineage. If duplicate genes originated through a genome duplication, then one might expect similar genomic neighborhoods for these genes (i.e., similar genes found adjacent to different ins genes), as observed for the duplicated proglucagon (gcg) and glucagon receptor (gcgr) genes of teleost fish (Irwin, 2014; Irwin & Mojsov, 2018). For the three insulin paralogs examined here, only very limited similarity was seen in the genomic neighborhoods (longer genomic regions were examined, with immediate neighborhoods summarized in Supplementary Table S2). A gene similar to tenm2 (tenurin transmembrane protein 2) was found adjacent to insa genes in Exox lucius, O. kisutch, O. mykiss, Paramormyrops kingsleyae, Salmo salar, and Sclerophages formosus, and insb genes in Pygocentrus nattereri, Salvelinus alpinus, and Sinocyctlocheilus rhinoceros (Supplementary Table S2). This might suggest that the insa and insb genes originated via the teleost-fish specific genome duplication. However, as the linkage between the tenm2 and ins genes was only observed in a small number of species, this would require large number of independent losses (or genomic rearrangements) of tenm2-like genes to yield the absence of a linked tenm2-like with most ins genes, therefore the evidence for the origin of insa and insb by a genome duplication event is not strong. No genes near insc were similar to those adjacent to insa or insb, thus it more likely originated as a single gene duplication event rather than through a genome duplication. If the insa and insb genes originated via a genome duplication, an addition single-gene duplication would still be needed to explain the origin of insc. The gene neighborhood data does not help resolve the relationships among the three ins genes. Intriguingly, the genomic neighborhoods of none of the insulin gene paralogs in teleost fish is similar to the typical vertebrate insulin gene neighborhood, where insulin is flanked by insulin-like growth factor 2 (igf2) and tyrosine hydroxylase (th) genes (Patton et al., 1998), although similar neighborhoods were found for the insulin genes in cartilaginous and lobe-finned fish (Supplementary Table S2). This suggests that the insulin genomic neighborhood was rearranged on the ancestral ray-finned fish lineage.

Orthologues genes often have similar genomic neighborhoods, thus can these neighborhoods can be used to assess orthology when gene sequence similarity is limited (Kurokawa et al., 2005). When the genomic neighborhoods surrounding insb and insc genes were examined (Figure 3 and Supplementary Table S2) shared features were identified for each gene. All insb genes are adjacent to a hmmr (hyaluronan mediated motility receptor)-like gene, while all insc genes are flanked by prr12 (proline rich 12) and scaf1 (SR-related CTD associated factor 1)-like genes (as shown for the Clupea harengus insb and Danio rerio insc genes in Figure 3), suggesting that their genomic neighborhoods are largely conserved since they originated in the early teleost. In contrast, several different genomic neighborhoods were observed for the insa genes (Supplementary Table S2, examples shown in Figure 3). The most phylogenetically widespread genomic neighborhood has a nipal (magnesium transporter NIPA3-like)-like gene adjacent to the insa gene (Supplementary Table S2) and is found in species representing Osteoglossomorpha (e.g., Sclerophages formosus, shown in Figure 3), Protacanthopterygii (e.g. Esox lucius and Salmo salar), Paracanthopterygii (e.g., Gadus morhua), and Percormorpha (e.g., Takifugu rubripes and Oryzias latipes). The only major group of teleost fish that does not have an insa gene adjacent to nipal are those of Ototomorpha (e.g., Danio rerio, shown in Figure 3), suggesting a change in the gene order occurred in this group and that the linkage of insa with nipal is ancestral. Within Percomorpha (Supplementary Table S2), the insa genes are found adjacent to nipal-like genes (as in Sclerophages formosus, Figure 3), while the insab genes are near a sh3pd2b (SH3 and PX domains 2B)-like genes (e.g., Haplochromis burtoni, Figure 3). The linkage of the insab genes with sh3pd2b strengthens the conclusion that all insab genes are orthologous and indicates that insa is located at the ancestral genomic location and was the source (locus-of-origin) for the insab gene that was inserted into a new genomic locus.

Figure 3 Genomic neighborhoods surrounding fish insulin genes

Examples of genomic neighborhoods found for insulin paralogs in ray-finned fish. Genomic neighborhoods are listed in Supplementary Table S2. A: Typical genomic neighborhood for insa and insaa genes as illustrated from Sclerophages formosus. B: Genomic neighborhood for insa genes in Danio rerio and close relatives. C: Typical genomic neighborhood for insab genes as illustrated from Haplochromis burtoni. D: Typical genomic neighborhood for insb genes as illustrated from Clupea harengus. E: Typical genomic neighborhood for insc genes as illustrated from D. rerio. See Supplementary Table S2 for full gene names.
Lineage-specific changes in insulin gene number

The gene duplication events described above explain most, but not all, of the insulin genes identified (Figure 1 and Supplementary Table S1). Many of the additional genes can be explained by tandem gene duplication, which lead to two insulin genes arranged head-to-tail in the genome (see Supplementary Tables S1, S2) and includes the insa1 and insa2 genes in Parmormyrops kingsleyae and in Larmichthys crocea, the insaa1 and insaa2 genes in Hippocampus comes, and the insab1 and insab2 genes in Boleopthalmus pectinirostris. Both Cyprinus carpio and Sinocyclocleithus rhinocerus have tandemly arranged insb genes (insb3 and insb4 in C. carpio and insb2 and insb3 in S. rhinocerus, Supplementary Table S2) that might have originated prior to the divergence of these two species (although, they would then have to be lost in the two other Sinocyclocleithus species). Sinocyclocleithus anshuiensis has two pairs of tandemly arranged genes, insa1/insa2 and insa3/insa4, which either originated prior to this species genome duplication (and then were lost in the closely related species that share this genome duplication), had parallel tandem duplications, or is an assembly error (the tandemly arranged genes are identical in sequence). The ins1 and ins2 genes of Lepisosteus oculatus might also have originated via a tandem duplication, which was then followed by rearrangements so that these lineage-specific gene duplicates are now found in opposite orientations on either side of a hmr-like gene (see Supplementary Table S2). More complex duplication events, resulting in genes moving to new locations are need to explain the remaining genes, with some being extremely recent (e.g., the identical insa1 and insa2 genes in Labrus bergylta) or more ancient (e.g., insa1 and insa2 gene of Esox lucius).

While duplication of genomic DNA is a frequent mechanism for the origin of duplicate genes, they can also be generated by retroposition of cDNA generated from mRNA transcripts (Long et al., 2013). Indeed, the well-characterized duplicate ins1 gene in mice was generated from an incompletely processed mRNA transcript (Shiao et al., 2008; Soares et al., 1985). Typically, insulin genes contain two coding exons that must be spliced together to create an intact coding sequence. Here I identified 6 insulin genes that are likely generated by reverse-transcribed mRNAs, as these genomic sequences do not have an intron interrupting the coding sequence (see Supplementary Figure S4 for an example). The 6 retro-processed insulin genes can be explained by three integration events: (1) between ankrd6 (ankyrin repeat domain 6) and lyrm2 (LYR motif containing 2)-like genes on the Lepisosteus oculatus lineage to generate ψins; (2) within an apbb3 (amyloid beta precursor protein binding family 8 member 3)-like gene on the Monopterus albus lineage to generate ψinsb; and (3) within a psd3 (PH and SEC7 domain containing protein 3)-like gene on the ancestral lineage for salmonids (Oncorhynchus mykiss, O. kisutch, Salmo salar, and Salvulinus alpinus) and the Northern pike (Esox lucius) that generated ψinsa in O. kisutch, S. salar, and S. alpinus (but was not found in O. mykiss) and an intact coding sequence (insa2) in Esox lucius (Figure 1 and Supplementary Figure S4 and Table S2). The maintenance of an intact open reading frame in Esox lucius insa2 (Supplementary Figure S4) suggests that this retropositioned sequence, like the mouse ins1 gene (Soares et al., 1985), is still functional. However, this sequence has lost its coding potential in salmonid fish and is a pseudogene (Figure 1 and Supplementary Table S1). Additional processed genes might also exist, as several incomplete genes (e.g., insb4 from Cyprinus carpio and insa3 from Parmormyrops kingsleyae) were identified (see Supplementary Table S1) that were located at unique location of the genome (see Supplementary Table S2) consistent with being inserted processed cDNAs, but the sequences were similar to either exon 1 or 2 (and not both), thus cannot be distinguished from a sequence generated by an DNA-mediated incomplete gene duplication event.

Gene for insulin a (insa) is evolving under greater evolutionary constraint

Our genomic and phylogenetic analyses of fish genome sequences demonstrated that teleost fish have three paralogous insulin genes. In contrast, most other vertebrate species only have one (Chan & Steiner, 2000; Conlon, 2000b). The increased number of insulin genes in teleost fish raises the possibility that they have been diversified to: (1) subfunctionalize distinct functions of insulin, (2) neofunctionalize to acquire new functions, or (3) lose function (pseudogenize). As a first step to explore possible changes in the biological roles of these distinct insulin genes I assessed the selective pressures acting upon the sequences. This can be done by comparing the rates of nonsynonymous (dN) to synonymous (dS) substitutions (Yang & Bielawski, 2000). If the protein encoded by a gene has lost its function, then it would be expected that there would be no selection against nonsynonymous substitutions, and that rates of nonsynonymous and synonymous substitutions would be the same rate (i.e., dN/dS=1, neutral evolution). If the protein encoded by these genes still had a function, then selection should act against a subset of nonsynonymous substitutions (the deleterious mutations) and be lower than the synonymous rate (i.e., dN/dS<1, purifying selection). Rarely, one might see dN/dS>1, which would indicate positive selection for change in amino acid sequence (Yang & Bielawski, 2000). If protein coding sequences of genes are being maintained for different functions, with different parts of the sequence being important for these functions, then one might see differing levels of selective constraint.

I used the program RELAX (Wertheim et al., 2014) to assess the levels of selective pressure (dN/dS) for each of the three insulin gene paralogs, and to determine whether the selective pressure was intensified or relaxed compared to the other insulin genes, with sequences from a non-teleost ray-finned fish, Lepisosteus oculatus, used as outgroup. When all sequences were analyzed, insa genes showed the stronger levels of purifying selection (dN/dS=0.2666, 0.3332, and 0.3258 for insa, insb, and insc, respectively; Table 1). If I restricted these analyses to either species that have all
three paralogs, or species that only have a single copy of these paralogs, to attempt to minimize species-specific effects (changes seen in species that only have some of the genes) or due to differences in gene number, the difference in the selective constraints acting on insa vs. insb and insc became even more pronounced (Table 1). The results from RELAX also indicated that selection intensification, compared to the other insulin sequences, occurred on insc, while selection relaxation occurred for insb (Table 1). No significant change in selection intensity was seen for insc vs. all other insulin sequences. Similar results, with insa showing greater constraint, were seen in comparisons of insa and insb genes in species that had both of these genes (Table 1). These results suggest that the insa paralog is under greater purifying selective constraint than either the insb or insc paralog, but also importantly demonstrate that both insb and insc are continuing to experience purifying selection. Thus, it can be concluded that the protein coding sequence of insa is under the greatest level of selective constraint, however, the coding sequences of both insb and insc are also experiencing selective constraints consistent with their protein products having essential biological functions.

Table 1 Differences in the selective pressure (dN/dS) acting on insulin paralogs in teleosts

| Sequences tested | insa | insb | insc |
|------------------|------|------|------|
| All              | 0.2666 (80) | 0.3332 (44) | 0.3258 (10) |
| Species with insa, insb, and insc | 0.2120 (11) | 0.3309 (9) | 0.3406 (9) |
| Species with single copy insa, insb, and insc | 0.1911 (4) | 0.3164 (4) | 0.3129 (4) |
| Species with single copy insa and insb | 0.1664 (17) | 0.3171 (17) | N/A |
| Species with single copy insa and insb, but no insc | 0.1366 (13) | 0.3191 (13) | N/A |

Numbers in brackets is the numbers of sequences used. a: The two complete Lepisosteus acaulatus ins sequences were used as outgroups. b: Test for selection intensification was significant. c: Test for selection relaxation was significant. N/A: Not applicable.

Adaptive evolution of insulin genes

While the protein products of the three insulin genes are being maintained for function, they might not be the same function. To examine whether any of the sequences might have gained new functions I tested for evidence of positive selection on lineages using aBSREL (Smith et al., 2015). Only one lineage showed significant evidence for episodic diversifying selection, the ancestral lineage for the insc genes. Inspection of the phylogenetic tree generated from all of the insulin coding sequences suggest that the ancestral branch for the insc genes is longer than for the insa and insb genes (Figure 2 and Supplementary Figure S2), consistent with a greater number of nonsynonymous substitutions driven by positive selection. No evidence for positive selection was found on the ancestral lineages for the insa or insb genes. These results might suggest that insc has acquired a new function (neofunctionalization) driven by positive selection, while insa and insb have been retained due to subfunctionalization of insulin functions between the two genes.

Relaxed selection on the insab duplicate of the insulin a (insa) gene

An intriguing observation from our analysis of the selective constrains acting upon insa, insb, and insc paralogs was that the calculated value for the selective constraint acting on insa varied considerably depending upon which sequences were used for the analysis (Table 1). The ratio of the nonsynonymous to synonymous rates was higher when all insa sequences were used than if sequences were used only from species that had all three paralogs or had only single copies of the insa and insb paralogs (Table 1). These observations suggest that inclusion of insa sequences from species that have multiple insa sequences yields higher estimates of the dN/dS ratio due to some of these sequences having lower levels of sequence constraint. As the duplication to generate the insaa and insab genes is a major source of the multiple insa genes in teleost fish (see Figures 1, 2 and Supplementary Figure S2 and Table S1), I compared the selective constraints acting upon insaa and insab genes (Table 2). The insaa coding sequences were found to be under selective constrains (Table 2) similar to those of other insa sequences, and especially those from species that had single copy insa, while the insab sequences displayed the lowest levels of constrains seen for any fish ins sequence (Tables 1, 2). Test for intensification or relaxation of selective constraint showed that insaa was under significant intensification of selective constraint, while insab was significant relaxation of selective constraint was demonstrated for insab (Table 2). These results suggest that insaa retains the function of insa, which would be consistent with it being at the locus of origin, while insab is evolving with far less constraint, to the extent that it has been lost on a number of lineages (e.g., Oreochromis niloticus, Fundamilla nyererei, M. zebra, and species of the genus Poecilia, see Figure 1). This might suggest that insab is not essential.

Table 2 Differences in the selective pressure (dN/dS) acting on insaa and insab genes

| Sequences tested | insaa | insab |
|------------------|-------|-------|
| All insa         | 0.1691 (14) | 0.4766 (9) |
| Species with one copy of each | 0.1251 (9) | 0.5139 (9) |

Numbers in brackets is the numbers of sequences used. a: The two complete Lepisosteus acaulatus ins sequences were used as outgroups. b: Test for selection intensification was significant. c: Test for selection relaxation was significant.
Processing of proinsulin sequences
To be functional, the protein product encoded by insulin genes need to be proteolytically processed and secreted to generate two-peptide chain insulin molecules (Steiner et al., 1996, 2009). Our analysis was initially only focused on sequences that showed similarity to previously characterized proinsulin sequences and had intact coding sequences, i.e., had an initiation codon, a termination codon and intact open reading frame with greater similarity to proinsulin than to other insulin-like sequences (e.g., insulin-like growth factors). To determine whether the encoded protein sequences could be secreted and properly processed I searched for potential signal peptidases and proprotein processing sites using programs that predict these sites (Duckert et al., 2004; Petersen et al., 2011; Southey et al., 2006).

The coding sequences of intact proinsulin open reading frames from all ray-finned fish identified here are predicted to have functional signal peptides (Table 3 and Supplementary Table S3 and Figure S6), thus should be able to be secreted. In addition, all of the proinsulin protein sequences encoded by the insa, insab, insb, and insc genes have potential prohormone protease cleavage sites that could yield typical two-chain insulin hormone molecules (Table 3 and Supplementary Table S3 and Figure S6). In contrast, however, only one of the 14 proteins encoded by insab genes is predicted to potentially produce a two-chain insulin (Table 3). For the insab sequences, only 2 have potential B-chain/C-peptide processing site and 9 have potential C-peptide/A-chain processing sites (Table 3 and Supplementary Table S3 and Figure S6), indicating that neither is conserved. Both NeuroPred (Southey et al., 2006) and ProP (Duckert et al., 2004) predicted similar sites, but ProP also provided a list of other potential processing sites that did not score well enough to be predicted sites (see Supplementary Table S3). The alternative potential sites identified by ProP in the insab proteins still would not generate two-chain insulin molecules similar to functionally characterized insulin molecules (Steiner et al., 1996, 2009) as they would generate very short B-chains, often missing residues essential for function.

Table 3 Secretion and processing of fish proinsulin proteins

| Gene | Signal peptide | B-C junction | C-A junction | 2-chain insulin |
|------|----------------|--------------|--------------|----------------|
| insa | 35/35<sup>a</sup> | 35/35 | 35/35 | 35/35 |
| insaa | 32/32 | 32/32 | 32/32 | 32/32 |
| insab | 14/14 | 2/14 | 9/14 | 1/14 |
| insb | 45/45 | 44/45 | 45/45 | 44/45 |
| insc | 10/10 | 10/10 | 10/10 | 10/10 |

<sup>a</sup>: Sequences with signal peptidase processing sites. <sup>b</sup>: Sequences with proprotein processing sites for the B-chain/C-peptide and C-peptide/A-chain processing sites. <sup>c</sup>: Number that can be secreted and properly processed to yield two-chain polypeptides. <sup>d</sup>: Number with the site/total number of sequences.

The hormone insulin is a two-chain peptide molecule held together by disulfide bridges (Steiner et al., 1996, 2009). To confirm that two-chain molecules could be produced from the proinsulin protein sequences I generated consensus sequences for the A- and B-chains for the different types of insulins (Figure 4). The lengths of the predicted A- and B-chains were similar between the different types of insulins, with more variation, at both the N- and C-termini, within proteins encoded by a type of gene than between types of genes (Figure 4 and Supplementary Figure S6). When cysteine residues were examined one of the two insc sequences from Sinocyclocheilus grahami (insc2) had a cystine replaced by an arginine residue. It is possible that the S. graham insc2 gene does not encode a functional insulin, but this is compensated by the presence of an insa<sub>1</sub> gene that encodes an insulin that can be secreted processed and has all six cysteine residues. Surprisingly, when I examined A- and B-chain sequences homologous to those of other insulins in the protein encoded by insab genes, all of the cysteine residues were perfectly conserved, however much of the rest of the sequence showed greater variation than within other types of insulins (Figure 4 and Supplementary Figure S6). This raises the possibility that insab could still fold in a similar fashion as typical insulin, thus be transported to insulin secretory granules, but then not be processed into an active hormone (Liu et al., 2015).

DISCUSSION
Searches of diverse fish genomes, including in the extensively characterized zebrafish (Danio rerio) genome, unexpectedly revealed the existence of three insulin gene paralogs (Figure 1 and Supplementary Figure S1 and Table S1). Previously, two paralogous insulin genes, insa and insb, had been found in the zebrafish genome that have distinct expression patterns (Irwin, 2004; Papasani et al., 2006). Here I found insa genes in the genomes of all teleost fish (Figure 1 and Supplementary Figure S1 and Table S1), which encode the previously isolated insulin hormone sequences (Caruso & Sheridan, 2011; Conlon, 2000b) that regulate glucose metabolism. Given the central role of insulin in the regulation of metabolism (Caruso & Sheridan, 2011; Röder et al., 2016; Weiss, 2009), it is not surprising that this gene was found in all fish. The presence of a high selective constraint (Tables 1, 2) on the protein sequences encoded by insa and insaa genes supports the conclusion that this sequence is essential. A surprising discovery was the presence of a duplicate of the insa gene, the insaa and insab genes, in a large number of teleost fish (Figure 1 and Supplementary Table S1). The protein coding sequence encoded by insab genes have the highest nonsynonymous substitution rate (Table 2), and thus least selective constraint, yet the consensus sequences of the potential A- and B-chains of insulin predicted from these genes have perfectly conserved cysteine residues (Figure 4). This suggests that these genes might encode proinsulin-like proteins that could properly fold but would not be properly processed in secretory granules to produce active insulin molecules (Liu et al., 2015). Thus, insab proteins might compete with insaa proteins for processing enzymes and modulate the release of functional insulin molecules in these species.
Consensus sequences for the A-chain (A) and B-chain peptides (B) for insulin encoded by \textit{insa}, \textit{insaa}, \textit{insab}, \textit{insb}, and \textit{insc} genes. Consensus sequences were generated from aligned predicted protein sequences (see Supplementary Figure S5) of genes with complete open reading frames (\textit{Larimichthys crocea insa2} was removed as it may not be functional). Height of the residue is proportional to representation among the compared sequences, while residue width represents proportion of sequences without a gap (thin residues are gaps in many sequences).

The \textit{insb} gene was found in the genomes of almost all teleost fish species examined, demonstrating that it is conserved in these fish (Figure 1 and Supplementary Figure S1 and Table S1). The selective forces acting on \textit{insb} are weaker than those acting on \textit{insa} (Table 1) suggesting that these two genes have different functions. Previous work indicates
that \textit{insb} is not appreciably expressed in the pancreas, and instead is more predominantly expressed in early development (Hrytsenko et al., 2016; Irwin, 2004; Papasani et al., 2006). The analysis of selective constraints and these expression results are consistent with speculation that \textit{insb} is not primarily involved in the regulation of blood glucose level, but instead has a role in development (Hrytsenko et al., 2016; Papasani et al., 2006). Given the conservation of the \textit{insb} gene across teleost fish, it likely acquired this role soon after its origin and has been conserved.

Unexpectedly I found an \textit{insc} paralog, and it was present in the well characterized zebrafish (\textit{D. rerio}) genome (Figure 1 and Supplementary Figure S1 and Table S1). Upon closer examination, the \textit{insc} gene in both the Ensembl (gene ID: ENSDARG00000096862) and NCBI (GenBank accession No.: 100534937) zebrafish genomes fail to predict an intact open reading frame, with the intact \textit{insc} coding sequence only coming from the linked mRNA sequence (XM_009299616.3) in the NCBI database (see Supplementary Table S1). I could find no raw sequence data (e.g., ESTs) that supports the existence of an intact open reading frame for zebrafish \textit{insc}, as all mRNA derived sequences (GenBank accession Nos. EH538807.1 and EH507658.1, forward and reverse sequences of the same EST clone) also contained the deletion found in the genomic sequence. This suggests that the zebrafish \textit{insc} gene does not encode an intact protein product. However, our analysis of the selective forces acting upon the \textit{insc} coding sequences indicate that the nonsynonymous rate of substitutions ($d_N$) is much lower than the rate of synonymous substitutions ($d_S$) consistent with selection to maintain a protein coding sequence (Table 1) in most, if not all, species that have intact \textit{insc} genes. The \textit{insc} gene, in contrast to \textit{insa} and \textit{insb}, is restricted to the genomes of only a few lineages, with this gene been lost from the genome of most teleost fish (Figure 1 and Supplementary Table S1). This distribution of the gene among fish suggests that it does not encode a function that is essential in most species, and its loss might have been compensated by the presence of paralogs of insulin in the fish genomes. Expression of \textit{insc}, based on a single EST clone (with sequences from both ends) that was identified in zebrafish, is found in the “gut and internal organs” of adults, thus potentially it could be expressed in the pancreas and be replaced by \textit{insa}. Further studies on the expression of \textit{insc} in other fish are needed to identify its role in physiology.

**CONCLUSION**

Searches of ray-finned fish genomes have demonstrated that they contain more insulin genes than previously appreciated and suggest that the roles of these genes have diversified. The diversification of the functions of insulin parallels the diversification of the proglucagon-encoded peptides found in fish (Irwin & Mojsov, 2018). Similarly, parallel changes in the biological functions of insulin and proglucagon-derived peptides have been observed in hystricomorph rodents (i.e., guinea pig and relatives), where changes in sequence and action of insulin have compensating changes in glucagon (Seino et al., 1988).

**COMPETING INTERESTS**

The author declare that they have no competing interests.

**AUTHORS’ CONTRIBUTIONS**

D.M.I. designed the study, conducted the analyses, and wrote the manuscript.

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