Pipefish Pigmentation Analysis

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19 September, 2019

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knitr::opts_knit$set(root.dir='../results/', fig.pos='H')
```

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library(knitr)
library(pander)
```

The purpose of this analysis is to compare the pigmentation pathways in syngnathids to look for potential genes that have diverged and may explain the variation in coloration in the family. Fish have up to six different types of pigmentation cells, which are derived from neural crest cells. Pigmentation cells fall into two categoires: light absorbent (melanophores, xanthophores, erythrophores, and cyanophores) and light reflective (iridophores and leucophores). Cyanophores are restricted to callionymid fishes (I. Braasch, Volff, and Schartl 2008), so are unlikely to be relevant to syngnathids. Leucophores have been identified in medaka, guppies, Japanese flounder, darkbanded rockfish, and killifish (H. A. M. Nagao Yusuke AND Takada 2018), but they may be more widespread than currently known. Melanophores, xanthophores, and iridophores are the most commonly-studied pigmentation cell types, largely because they are the only three pigmentation cells found in zebrafish. Despite this wide taxonomic variation in pigmentation cells, the genetic pathways involved in the differentiation and proliferation of pigmentation cells are highly conserved, and many are orthologous to mammalian pigmentation genes. In a study of poison dart frogs, key differentially expressed genes include those involved in melanogenesis, melanocyte differentiation, melanocytype proliferation and purine synthesis and iridophore development (Stuckert et al. 2018).

Genome duplication events in the teleost lineage have allowed for subfunctionalization and thus the diversification of pigmentation patterns. In some groups, genes have been duplicated further (I. Braasch, Volff, and Schartl 2008); and the duplication of *slc* genes in the vertebrate genome has been described as critical to the production of all six kinds of pigment cells (Kimura et al. 2014). Several pigmentation genes apparently don't have duplicates, e.g. *oca2*, *slc45a2*, *slc24a5*, and *mc1r*. However, key genes such as *mitf*, *sox10* and *csf1r* have been duplicated and have clearly subfunctionalized; for example, *mitfa* effects neural crest derived pigmentation wherease *mitfb* affects retinal pigment epithelium. Although the key genes appear to be conserved across fishes, we expect complementary gene changes across species, and indeed zebrafish and medaka have marked differences in both the copies of genes present and the functions of genes (for example, *sox10a* has been lost from zebrafish but not medaka). Additionally, examples of lineage-specific gene duplications exist in Xiphophorus and salmonids (I. Braasch, Volff, and Schartl 2008).

In zebrafish (and likely other sexually dimorphic fish) there are two stages of chromatophore development: embryonic and metamorphic stages. In zebrafish, in which iridophores are the first pigment cells to differentiate (D. M. Patterson Larissa B. AND Parichy 2013), the juvenile pattern of iridophore cells may act as the prepattern to form the complete adult stripes (Kondo and Watanabe 2014). Some zebrafish mutants have normal embryonic stages of development but deficiencies in metamorphic melanophores, suggesting that different mechanisms may play a role in these two different stages of colour pattern development (D. M. Patterson Larissa B. AND Parichy 2013). Little work has been done in this realm and much is still unknown.

In all pigment cells, there are several key steps that use different genetic mechanisms: migration to the skin, differentiation into a specific pigmentation cell type, proliferation (mediated by communication with other pigmentation cells), and production of the pigment itself. These processes have been primarily studied in zebrafish, with some complementary work also done in medaka, so the genes involved have primarily been identified in those two species. This provides a starting point for our analyses, but as you will see below, differences have been observed between zebrafish and medaka, so the mechanisms are likely similar but may differ in additional species (Kondo and Watanabe 2014).

Migration

Pigment cells originate from neural crest-derived stem cells, and their progenitors must migrate to the skin surface. Pigment cells migrate in an unpigmented state (known as chromatoblasts) (Kelsh 2004). A key characteristic difference among the three pigment types is that melanophores hardly every divide and migrate, whereas iridophores and xanthophores can proliferate and spread in the skin (Nüsslein-Volhard and Singh 2017). Additionally, melanoblasts can become other pigment cell types, a feature which is not true for the other pigment cell progenitors. These melanophore precursors express *mitfa*, a gene which is required for melanophore proliferation. The precursors to iridophores are located beside the dorsal root ganglia and differentiate as they migrate to the body surface. In zebrafish, xanthophores require iridophores to develop in the trunk region of the skin (D. M. Patterson Larissa B. AND Parichy 2013).

In zebrafish, the location of some adult melanophores and iridophores are established during embryogenesis, dependent on *erbb3b*. This population of precursor chromatophores expands and is maintained in pre-metamorphic larvae dependent on *erbb3b* and *tuba8l3a*, and during metamophrosis these precursor cells differentiate into specific pigment cells. Adult pigment cell precursors migrate to the hypodermis during pigment pattern metamorphosis and extra-hypodermal, nerve-associated precursors (i.e., cells that could be associated with peripheral nervew or ganglia instead of skin colouration) are essential for metamorphic melanophore development (Budi 2011).

Differentiation

The first pigment cell to differentiate can have a critical influence in specifying the pattern (L. B. Patterson, Bain, and Parichy 2014). *Basonuclin-2* (*Bnc2*) is necessary for development and maintenance of melanophores – by regulating expression of *kitlga* – and xanthophores – by regulating *csf1*. The role of *bnc2* in iridophore development is less clear, as iridophores produce *csf1* and contribute to organizing melaonophores and xanthophores (D. M. Patterson Larissa B. AND Parichy 2013). The exact mechanisms for the differentiation process of different chromatophore types are not fully known, but mutant zebrafish and medaka lines that lack proper chromatophore expression have shed light on the key genes involved for melanophores, iridophores, xanthophores and leucophores:

Melanophores: In melanophore development, *sox10* plays a critical role through the transcriptional regulation of *mitfa*; *mitf* activity is required for melanophore proliferation and survival. Ectopic *mitfa* expression causes ectopic expression of the melanogenic enzyme *dct* and kit ligand (Elworthy et al. 2003), which along with *ednrb1* are key for melanophore development (Takahashi and Kondo 2008; Kelsh 2004). Melanophore number and differentiation – but not embryonic pigmentation – are impacted by *asip1*, which decreases *dct* and *tyrp1b* expression and inhibits melanophore differentiation, a phenomenon that is similar to what is described in mammalian species (R. M. Ceinos et al. 2014).

Iridophores: The specification of iridophores is dependent on *sox10* expression and its regulation of *mitfa* (H. A. M. Nagao Yusuke AND Takada 2018). Melanoblasts can result in iridophores instead of melanophores if *foxd3* is expressed and inhibits *mitfa* (Budi 2011), and regulation of *csf1a* gene expression is key to iridophore organization and expression (L. B. Patterson, Bain, and Parichy 2014). Iridophores also require leukocyte tyrosine kinase (*ltk*) for specification and *Mpv17*, a mitochondrial protein, for survival (Singh and Nüsslein-Volhard 2015). The development of the adult precursor cells relies on *erbb3b*, which migrate to the hypodermis during metamorphosis and require *tuba8l3a*, *kitla*, *ednrb1*, and *csf1r* for differentiation into iridophores (Budi 2011).

Xanthophores and Leucophores: The development and survival of xanthophores is dependent on *fms* (Kelsh 2004; Takahashi and Kondo 2008) and *pax7a* (Kimura et al. 2014). In medaka, which unlike zebrafish have leucophores, it has been demonstrated that xanthophores and leucophores share a neural crest cell lineage (Dooley 2015; Kimura et al. 2014; T. A. S. Nagao Yusuke AND Suzuki 2014). Given this shared lineage, a set of key genes are involved in determining the fate of chromatoblast cells towards either a leucophore or xanthophore fate: *slc2a15b*, *slc2a11b* (Kimura et al. 2014), *sox5*, (H. A. M. Nagao Yusuke AND Takada 2018). Same as with other chromatophore types, the locations of other chromatophore cells impacts

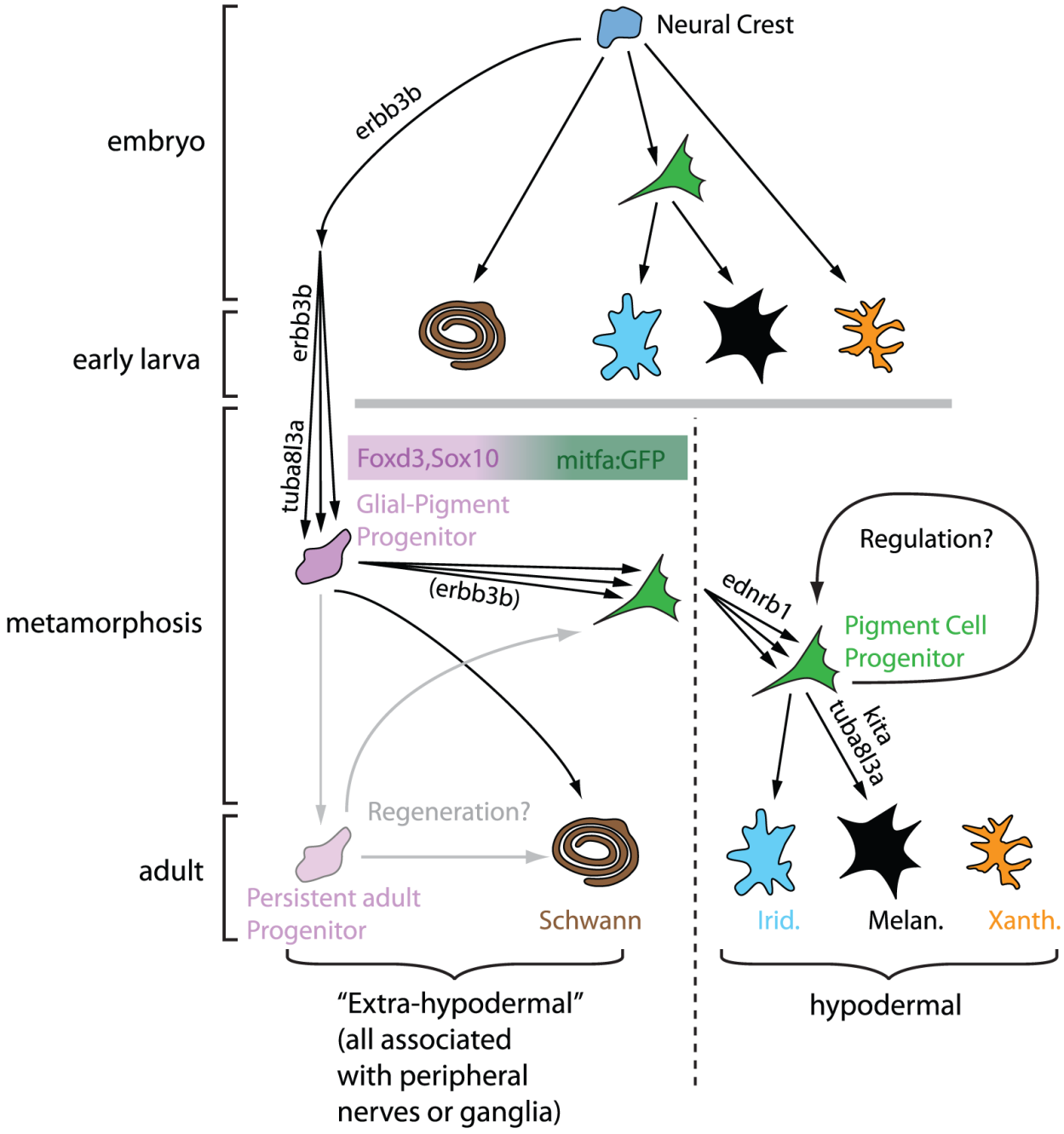

Figure 1: Pathways for chromatophore differentiation using neural crest cells, from Budi et al. (2011)

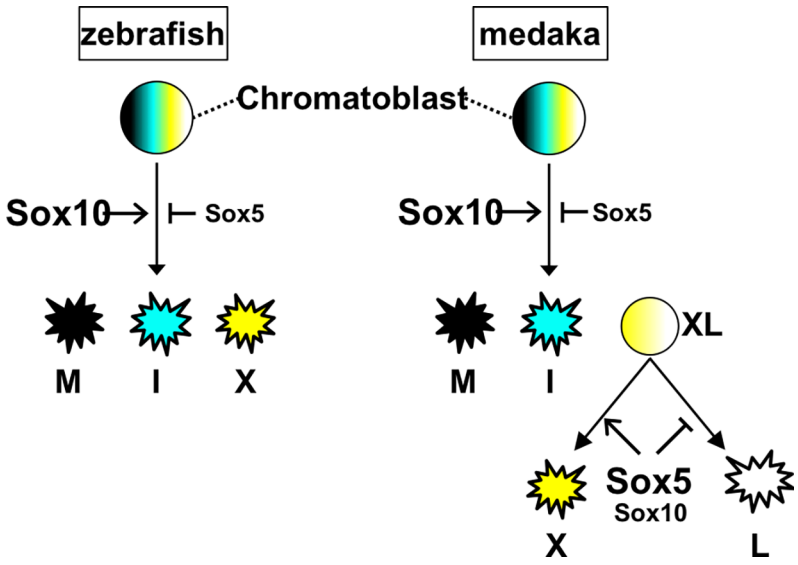

Figure 2: The roles of sox10 and sox5 in chromatophore differentiation. From Nagao et al. (2018)

the location of where these two pigment cell types differentiate; The presence of xanthophores prevents leucophores from being positioned laterally close to xanthophores (T. A. S. Nagao Yusuke AND Suzuki 2014). The mode of *sox5* is different in medaka than zebrafish: in zebrafish, *sox5* represses xanthophore development, whereas in medaka *sox5* promotes xanthophore specification and represses leucophores – indeed, cells that become xanthophores maintain *sox5* expression (H. A. M. Nagao Yusuke AND Takada 2018). *sox5* acts antagonistically against *sox10* in specification of all pigment cell lineages in zebrafish and only in melanophore and iridophore lineages in medaka (H. A. M. Nagao Yusuke AND Takada 2018).

Proliferation and pattern forming

Proliferation of pigment cells and the patterning of the skin is regulated by competitive interactions among pigment cells. These interactions are facilitated by direct cell-cell contact, likely involving potassium channels and gap junctions (including genes such as *kir7.1* (Singh and Nüsslein-Volhard 2015), *Kcnj13*, *Connexin 41.8*, *Connexin 39.4*, *spermidine*, and tight junction protein 1a *tjp1a* (Nüsslein-Volhard and Singh 2017; Takahashi and Kondo 2008)), but the actual underlying biochemical and molecular events remain highly conjectural (Nüsslein-Volhard and Singh 2017). Iridophores interact with xanthophores at the very least by providing a localized source of *csf1*, which promotes xanthophore development (D. M. Patterson Larissa B. AND Parichy 2013). Additionally, iridophores can influence melanophore patterning independently of interactions between xanthophores and melanophores (D. M. Patterson Larissa B. AND Parichy 2013).

Melanophores also influence their own patterning; melanophores repulse one another and disperse from the aggregated condition in the absence of xanthophores (Takahashi and Kondo 2008). Some of the aspects of melanophore behavior may be regulated by immunoglobulin superfamily member 11 (*lgsf11*). Melanocortin signalling is mediated by binding to a family of G protein coupled receptors that positively couple to adenyly cyclase, the five-ish melanocortin receptors (J. M. Cerdá-Reverter et al. 2011).

Xanthophores likely gather using the binding molecules such as N-CAM and N-cadherin, which in medaka are expressed in xanthophores but not melanophores or iridophores (FUKUZAWA and OBIKA 1995), though the expression of these molecules do not fully explain the aggregation patterns observed (Takahashi and Kondo 2008).

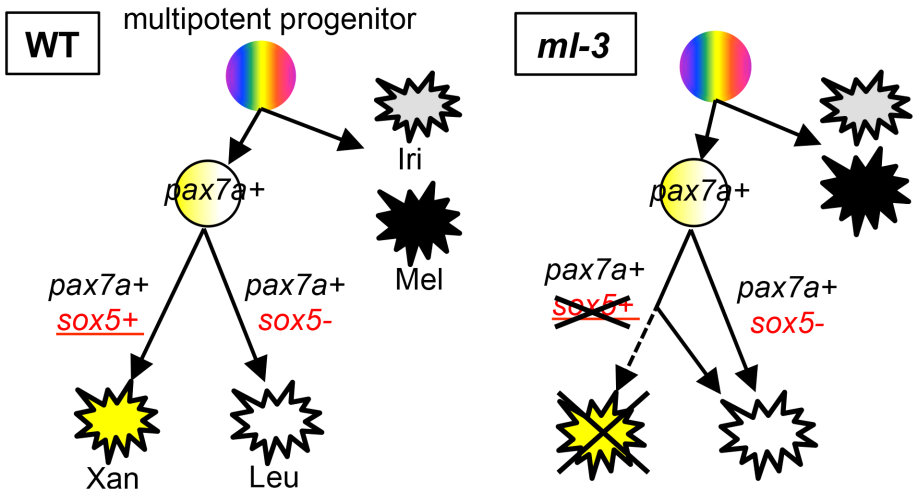

Figure 3: The roles of *pax7a* and *sox5* in leucophore and xanthophore differentiation in medaka. From Nagao et al. (2014)

Pigment production

Melanophore pigmentation depends on proopiomelanocortin (*pomc*), which encodes a complex protein precursor in the opioid/orphanin gene family. In tetrapods *pomc* is cleaved into three main domains: MSH, ACTH, and C-terminal-beta-lipotropin. The number of MSH domains varies in fish due to substitutions, deletions, and whole genome duplications. Zebrafish, medaka, stickleback, and pufferfish have two POMC genes (POMCa and POMCb), but three different forms have been found in other speices. In 5 species of African cichlids, *pomcb* was expressed in the skin (Harris, Dijkstra, and Hofmann 2014). In *Astatotilapia burtoni*, estrogen receptor sites were found in all three *pomc* paralogs and androgen receptor sites in two of the three (Harris, Dijkstra, and Hofmann 2014). Injecting male *A. burtoni* with *alpha*-MSH (one of the *pomca* peptides) increased the yellow colouration by causing the xanthophores to disperse (Dijkstra et al. 2017). However, the endogenous gene expression levels of the melanocortin system in *A. burtoni* are higher in blue males than yellow males; *mc1r* and *pomcb* were both more highly expressed in blue males than yellow males, though *asip1* was higher in yellow than blue males – an opposite expression pattern to a different yellow cichlid species (Dijkstra et al. 2017). The agouti-signalling protein (*asip*) and agouti-related protein (*agrp*) compete with melanocortin peptides by binding to melanocortin receptors (*mc1r*, *mc2r*, *mc3r*, *mc4r*, and *mc5r*) (J. M. Cerdá-Reverter et al. 2011). It is worth noting that *mc2r* and *mc5r* are found on the same chromosome in mammals, birds, and fish (Kumar 2011). *asip* is mainly expressed in the skin where it is involved in adult pigment patterning. Some of the melanocortin receptors are more receptive to *asip* and *agrp* than others; for instance, in mice, *agrp* strongly binds to *mc3r* and *mc4r* but not *mc1r*. In fish, there are two copies of each agouti protein – *asip1* and *asip2*, *agrp1* and *agrp2*. As seen before, these multiple copies have resulted in subfunctionalization: *agrp1* is expressed in the skin whereas *agrp2* is only expressed in the pineal glad of zebrafish, though expression patterns may be wider in salmon (J. M. Cerdá-Reverter et al. 2011).

A xanthophore-specific gene, *xdh*, gene encodes an enzyme required for the synthesis of yellow-orange pteridine (R. M. Ceinos et al. 2014), although *xdh* was detectable in both xanthophores and leucophores in medaka (T. A. S. Nagao Yusuke AND Suzuki 2014). In medaka *gch* is a marker for xanthophores and leucophores but not melanophores (though it is expressed in melanophores in zebrafish). The coloration of xanthophores and leucophores is in part encoded by a solute carrier protein, *slc2a15b* (H. A. M. Nagao Yusuke AND Takada 2018). The characteristic white coloration and auto-fluorescence of leucophores comes from uric acid (H. A. M. Nagao Yusuke AND Takada 2018).

The purine synthesis pathway is required for the pigmentation characteristic of iridophores and xanthophores (Ng et al. 2009). Iridophore pigmentation is caused by the enzyme *pnp4a* (Kimura, Takehana, and Naruse 2017), although the density of the pigment cells also influences the expressed pigmentation. There are three

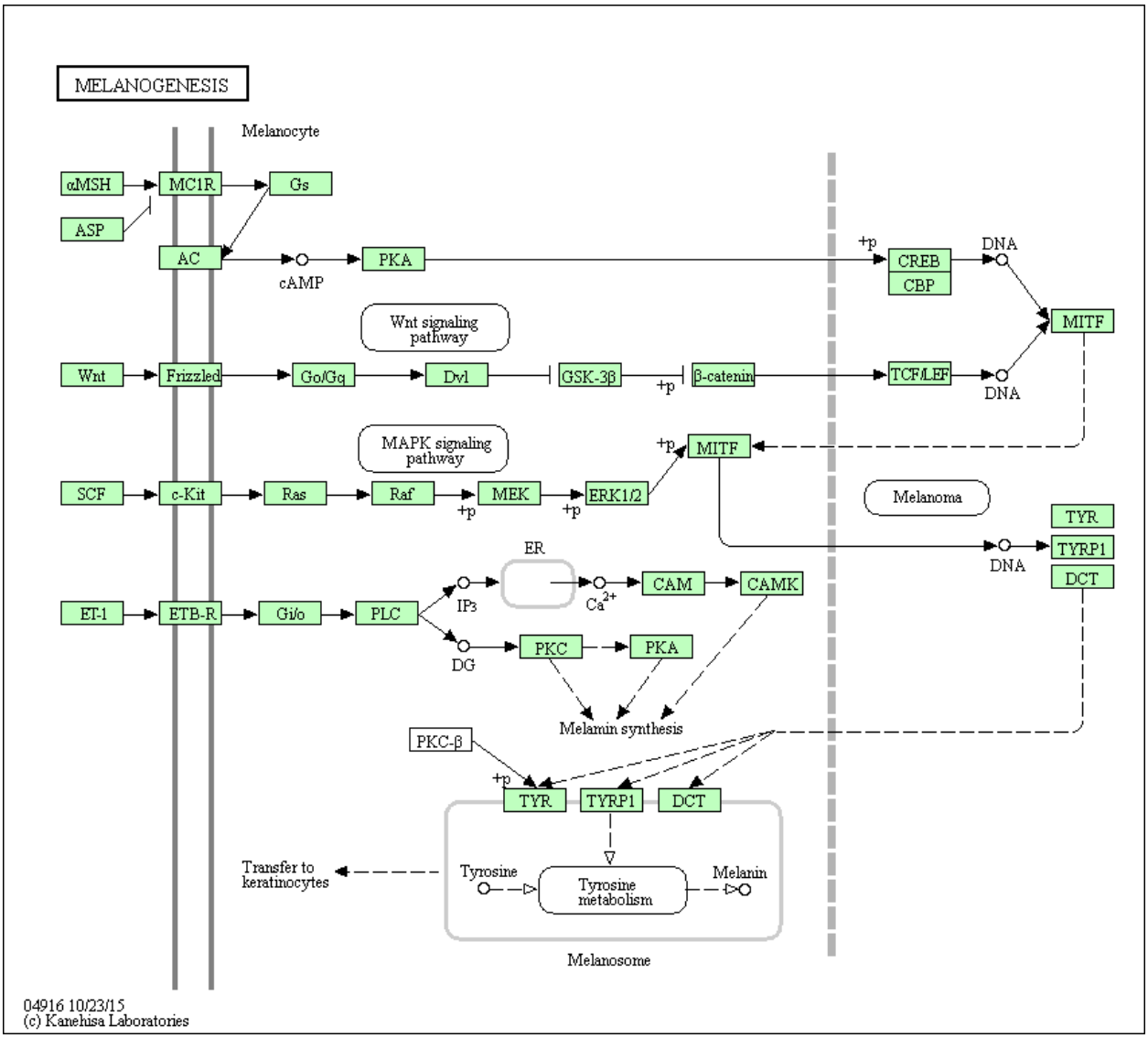

Figure 4: The melanogenesis pathway in *Danio rerio*

iridophore shapes (Singh and Nüsslein-Volhard 2015): *densely packed S (surface) iridophores* loosely packed iridophores resulting in the bluish coloration of zebrafish stripes *L-iridophores underneath the melanophore stripes The density of iridophores may be regulated by* ednrb1* and *ece2* (Singh and Nüsslein-Volhard 2015).

Two additional pigmentation mutants that are informative for understanding the pathways involved in pigment production: *gart* and *paics*. These mutants have nearly absent xanthophore and iridophore pigmentation in embryos and melanophores are substantially decreased. The causal mutations encode phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase (*gart*), a trifunctional enzyme that catalyzes steps 2, 3 and 5 of inosine monophosphate (IMP) synthesis and phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase (*paics*), a bifunctional enzyme that catalyzes steps 6 and 7 of this process (Ng et al. 2009).

All in all, similar genes seem to be underlying the different chromatophores but regulation of the genes seems to differ. (Cal et al. 2017) put together a hypothetical diagram for how the melanocortin pathway may influence fish pigmentation:

Identifying the candidate genes in the scovelli genome

First, I created a list of the genes that have been shown to play a causal role in colouration in fishes (reviewed above).

```
candidates<-read.csv("./pigmentation-evolution/candidate_genes.csv",stringsAsFactors = FALSE)
pander(candidates[,1:5], split.cell = 100, split.table = Inf)
```

Gene	Gene_name	Reason	species	citation
Agrp1	agouti-related protein 1	melanocortin antagonist	fish	(Cal et al. 2017)
Agrp2	agouti-related protein 2	melanocortin antagonist; responsible for formation of melanic strips in African cichlids	fish; cichlids	(Cal et al. 2017; Kratochwil et al. 2018)
aim1a	Absent-in-melanoma; crybg1; st4	melanosomal transporter	fish	(Braasch, Schartl, and Volff 2007)
aim1b	Absent-in-melanoma; crybg1; st4	melanosomal transporter	fish	(Braasch, Schartl, and Volff 2007)
Asip1	agouti-stimulating protein 1	melanocortin antagonist; involved in governing the ventral-specific expression of agouti and the dorso-ventral pigmentary pattern	fish; goldfish	(Cal et al. 2017; Cerdá-Reverter et al. 2005)
Asip2	agouti-stimulating protein 2	melanocortin antagonistt; involved in governing the ventral-specific expression of agouti and the dorso-ventral pigmentary pattern	fish; goldfish	(Cal et al. 2017; Cerdá-Reverter et al. 2005)
atic	atic	identified by differential expression between chromatophore types	zebrafish	(Higdon 2013)
bnc2	basonuclin-2	key in melanophore and xanthophore differentiation	zebrafish	(D. M. Patterson Larissa B. AND Parichy 2013)
ckit	receptor for tyrosine kinase	positive feedback with tfec and ltk driving iridophore specification	zebrafish	(Petratou 2018; Higdon 2013; Kelsh 2004)

Gene	Gene_name	Reason	species	citation
connexin39.4	gap junction protein 1a	cell-cell contact involved in stripe pattern formation	zebrafish	(Nüsslein-Volhard and Singh 2017)
cx41.8	leopard;connexin 41.8; gap junction protein alpha 5b	cell-cell contact involved in stripe pattern formation	zebrafish	(Watanabe et al. 2006)
csf1ra	Colony-stimulating factor receptor 1a; pfeffer/panther; fms; c-fms	melanophore stripes are broken up and separated by iridophores	zebrafish	(Frohnhöfer et al. 2013; I. Braasch, Volff, and Schartl 2008; Kelsh 2004; Takahashi and Kondo 2008)
dct	dopachrome tautomerase	melanophore specific gene	zebrafish	(Elworthy et al. 2003)
ece2	switch of loose iridophores to desne iridophores		zebrafish	(Singh and Nüsslein-Volhard 2015)
ece2	switch of loose iridophores to desne iridophores		zebrafish	(Singh and Nüsslein-Volhard 2015)
ednrba	endothelin receptor b1a/rose	mutants show a reduction of iridophores, a reduction of melanophores, and fewer stripes; loss of expression of iridophores in adults	zebrafish	(Frohnhöfer et al. 2013; D. M. Parichy et al. 2000; Higdon 2013)
erbb3b	receptor tyrosine-protein kinase erbB-3; HER3	determines location of melanophores and iridophores	zebrafish	(Budi 2011)
foxd3	forkhead box d3 transcription factor	repress mitfa expression biasing melanoiridoblast progenitors towards an iridophore fate; loss of this gene caused fewer iridophores	zebrafish	(Curran, Raible, and Lister 2009; Curran et al. 2010)
gart	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	catalyzes steps in inosine monophosphate synthesis	zebrafish	(Ng et al. 2009)
gch1	GTP cyclohydrolase I	precursor for pteridine pigments expressed in xanthophores and emlanophores in zebrafish but only in xanthophores and leucophores in medaka	medaka	(T. A. S. Nagao Yusuke AND Suzuki 2014; Braasch, Schartl, and Volff 2007)
kcnj13	Kir7.1; potassium voltage-gated channel subfamily j member 13; jaguar/obelix	cell-cell contact	zebrafish	(Nüsslein-Volhard and Singh 2017; Iwashita et al. 2006; Singh and Nüsslein-Volhard 2015)
kitlga	kit ligand a/kitla	melanogenic factor	zebrafish	(Budi 2011)
igsf11	immunoglobulin superfamily member 11	may regulate aspects of melanophore behavior	zebrafish	(D. M. Patterson Larissa B. AND Parichy 2013)

Gene	Gene_name	Reason	species	citation
ltk	leukocyte tyrosine kinase	mutants show a reduction of iridophores, a reduction of melanophores, and fewer stripes	zebrafish	(Frohnhöfer et al. 2013)
mc1r	melanocortin receptor 1	it's central to pigmentation variation in many fish	zebrafish	(Cal et al. 2017)
mc2r	melanocortin receptor 2	other melanocortin receptors may be involved in the other chromatophore types	zebrafish	(Cal et al. 2017)
mc3r	melanocortin receptor 3	other melanocortin receptors may be involved in the other chromatophore types	zebrafish	(Cal et al. 2017)
mc4r	melanocortin receptor 4	other melanocortin receptors may be involved in the other chromatophore types	zebrafish	(Cal et al. 2017)
mc5ra	melanocortin receptor 5	other melanocortin receptors may be involved in the other chromatophore types	zebrafish	(Cal et al. 2017)
mc5rb	melanocortin receptor 5	other melanocortin receptors may be involved in the other chromatophore types	zebrafish	(Cal et al. 2017)
mitfa	microphthalmia-related transcription factor a, nacre/mitf-related gene/mitf2	mutants lack melanophores completely	zebrafish	(Frohnhöfer et al. 2013; Petratou 2018; Curran, Raible, and Lister 2009)
Mpv17	mitochondrial protein	important for iridophores	zebrafish	(Singh and Nüsslein-Volhard 2015)
oca2	oca2	melanosomal transporters	fish	(Braasch, Schartl, and Volff 2007)
paics	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	catalyzes steps in inosine monophosphate synthesis	zebrafish	(Ng et al. 2009)
pax7a	paired box 7a	key in determining differentiation of xanthophores and leucophores	medaka	(Kimura et al. 2014)
pnp4a	pnp4a	a key differentiation gene driven by sox10 and tfec; causal gene causing iridophore reduction in larvae and adults	zebrafish; medaka	(Petratou 2018; Kimura, Takehana, and Naruse 2017)
pomca	proopiomelanocortin gene	melanocortin stimulating hormone (msh), adrenocorticotropic hormone, and c-terminal-beta-lipotropin derived from pomc (important in melanophore pigmentation)	fish	(Cal et al. 2017)

Gene	Gene_name	Reason	species	citation
pomcb	proopiomelanocortin gene	melanocortin stimulating hormone (msh), adrenocorticotropic hormone, and c-terminal-beta-lipotropin derived from pomc (important in melanophore pigmentation)	fish	(Cal et al. 2017)
pts	6-pyruvoyltetrahydropterin synthase	pteridine synthesis and xanthophore-based yellow color	guppy	(Ben et al. 2003; Braasch, Schartl, and Volff 2007)
slc24a5	solute carrier family 24 member 5	melanosomal transporters	fish	(Braasch, Schartl, and Volff 2007)
slc2a11b	solute carrier family 2 member 11	affect coloration of both xanthophore and leucophore lineages	medaka	(T. A. S. Nagao Yusuke AND Suzuki 2014)
slc2a15b	solute carrier family 2 member 15	required for coloration of xanthophores and leucophores	medaka	(T. A. S. Nagao Yusuke AND Suzuki 2014)
slc45a2	solute carrier family 45 member 2	melanosomal transporter	fish	(Braasch, Schartl, and Volff 2007)
sox10	Sry-related HMG-box 10	positive feedback with tfec and ltk driving iridophore specification	zebrafish	(Petratou 2018)
sox5	Sry-related HMG-box 5	determines if chromatoblasts become leucophores or xanthophores	medaka	(H. A. M. Nagao Yusuke AND Takada 2018; T. A. S. Nagao Yusuke AND Suzuki 2014; Kimura et al. 2014)
spermidine	spermidine	cell-cell contact	zebrafish	(Nüsslein-Volhard and Singh 2017)
tfec	transcription E factor	positive feedback with tfec and ltk driving iridophore specification	zebrafish	(Petratou 2018)
tjp1a	tight junction protein 1a	cell-cell contact	zebrafish	(Nüsslein-Volhard and Singh 2017)
tuba8l3a	tubulin alpha 8 like 3	influences determination of chromatoblasts	zebrafish	(Budi 2011)
tyr	tyrosinase	important in pigment production in melanophores	medaka	(H. A. M. Nagao Yusuke AND Takada 2018)
tyrp1a	Tyrosinase-related protein 1	melanogenic enzyme	zebrafish	(R. M. Ceinos et al. 2014)
tyrp1b	Tyrosinase-related protein 1	melanogenic enzyme	zebrafish	(R. M. Ceinos et al. 2014)
xdh	xanthine dehydrogenase	an enzyme required for pteridine synthesis and is characteristic of xanthophores	medaka	(T. A. S. Nagao Yusuke AND Suzuki 2014)

Gene	Gene_name	Reason	species	citation
sparc	secreted protein acidic and rich in cysteine	arranging and distributing cells in the extra-cellular matrix and is up-regulated in cases of melanoma where it increases motility of melanocytes; up-regulated in response to estrogen in S. scovelli	humans	(Robert et al. 2006)
scarb2	lysosome membrane protein 2	dilute melanosomes within melanocytes; up-regulated in response to estrogen in S. scovelli	zebrafish	(Navarro et al. 2008)

To make sure that we're working with the correct genes, we'll download sequences for other fish species from Ensembl and compare them to the *Syngnathus scovelli* genome using local blastn. We can find the genes using bioconductor and using the zebrafish ID symbols. We'll use the gene_exon, which should only include coding sequences.

```
source("http://bioconductor.org/biocLite.R")
biocLite("biomaRt")
```

```
library(biomaRt)
#ensembl = useEnsembl(biomart="ensembl",host = "www.ensembl.org")

# go through each species in the column names and then through each gene at the row
genomes<-grep("ensembl",colnames(candidates),value=TRUE)
genes<-unique(candidates$zfin_id_symbol)

matches<-lapply(genomes,useEnsembl,biomart="ensembl",host = "www.ensembl.org",mirror='uswest')

df<-lapply(as.character(genes),function(gene,matches,candidates){
  spp<-do.call(rbind,lapply(matches,function(genome,gene){
    print(paste(gene,"in",genome@dataset))
   if(class(try(getBM(attributes=c('ensembl_gene_id','zfin_id_id'),filters = 'zfin_id_symbol',
                      values = gene,mart=genome),silent=TRUE))=='try-error'){
     print(" was not found with getBM.")
     seqs<-data.frame(coding=NA, ensembl_gene_id=NA,gene=gene,dataset=genome@dataset )
    }else {
      info<-getBM(attributes=c('ensembl_gene_id',"description","chromosome_name",
                               "strand","start_position",
                               "end_position",'zfin_id_symbol','zfin_id_id'),
                  filters = 'zfin_id_symbol',
                  values = gene,mart=genome)
      print(" was found with getBM")
      if(class(try(getSequence(id=info$ensembl_gene_id,seqType = 'coding',
                               mart=genome,type="ensembl_gene_id"),silent=TRUE))!='try-error' &
         nrow(info)>0){

        seqs<-getSequence(id=info$ensembl_gene_id,seqType = 'coding',
                          mart=genome,type="ensembl_gene_id")
        seqs$gene<-gene
        seqs$dataset<-genome@dataset
        print(" and sequence was retrieved.")
```

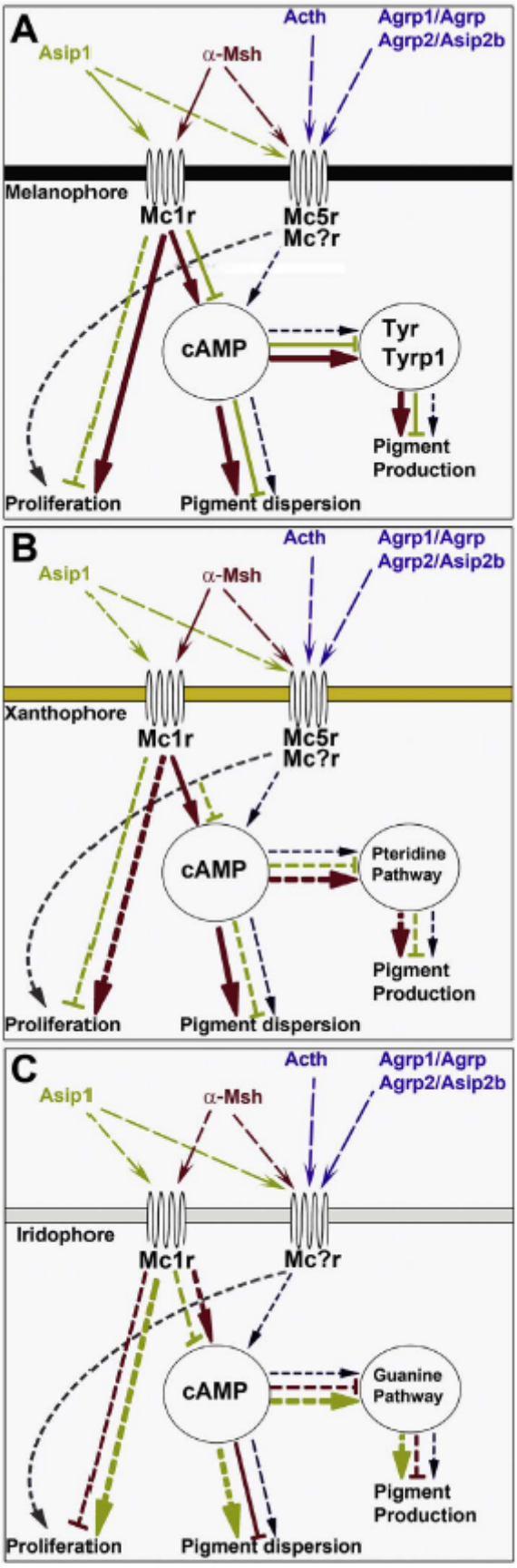

Figure 5: The putative key genes and proteins involved in melanophore, xanthophore, and iridophore proliferation and pigmentation production. From Cal et al. (2017)

```
      }else{
        print(" but sequence was not retrieved.")
        seqs<-data.frame(coding=NA, ensembl_gene_id=NA,gene=gene,dataset=genome@dataset )
      }
    }
   return(data.frame(seqs))
  },gene=gene))
},matches=matches, candidates=candidates)

#save the ensembl IDs to the candidates df
eids<-data.frame(do.call(rbind,lapply(df,function(data){
  ensemblids<-tapply(data$ensembl_gene_id,data$dataset,function(x){
    paste(unique(x),collapse=";")
  })
})),stringsAsFactors = FALSE)
eids$gene<-genes
cands<-merge(candidates[,!colnames(candidates)%in% colnames(eids)],eids,
             by.x="zfin_id_symbol",by.y="gene",all.y=TRUE)
write.csv(cands,"./pigmentation-evolution/candidates.csv")

rm(matches,ensembl) #save memory
#write sequences to fasta files
f<-lapply(df[which(genes!="")],function(gene.info){
  apply(gene.info,1,function(gene){
    if(!is.na(gene["ensembl_gene_id"])){
      browser()
      name<-paste(">",paste(gene["dataset"],gene["gene"],gene["ensembl_gene_id"],
                            sep="_"),sep="")
      filename<-paste("../data/gene_seqs/",
                      gsub("(\\w+)_gene_ensembl","\\1",gene["dataset"]),"_",
                      gene["gene"],".fasta",sep="")
      write.table(gene["coding"],filename,row.names=FALSE,quote=FALSE,col.names = name)
    }
  })
})
```

To do these comparisons, I ran blastn with the *S. scovelli* genome as a local blast database. Then I collated the blast matches using this R code:

```
candidates<-read.csv("./pigmentation-evolution/candidates.csv")
blast.files<-list.files(pattern = "*ssc.blast$",path="../data/gene_seqs",full.names = TRUE)
blast.dat<-do.call("rbind",lapply(blast.files,function(file){
  if(class(try(read.delim(file,comment.char="#",header=FALSE),silent=TRUE))=="try-error"){
    dat<-data.frame(rbind(rep(NA,12)),stringsAsFactors = FALSE)
  }else{
    dat<-data.frame(read.delim(file,comment.char="#",header=FALSE),stringsAsFactors = FALSE)
  }
  colnames(dat)<-c("qseqid", "sallseqid", "sallacc", "qstart", "qend", "sstart",
                   "send", "evalue", "bitscore", "length",
                   "gaps", "salltitles")
  dat$gene<-as.character(gsub(".*\\/?([A-z]+_)(\\w.*).ssc.blast","\\2",file))
  dat$query_spp<-as.character(gsub(".*\\/([A-z]+)_(\\w.*).ssc.blast","\\1",file))
  return(dat)
}))
```

Before annotating the genome with pigmentation pathway genes, I want to ensure that I'm focusing in on the truly best matches – genomic regions that are really good matches (low e-value, few gaps, and long alignment length) in multiple (ideally all) the species blasted here. I can do this in two steps: first by sorting the dataset in R and then by checking ones with conflicts by eye to find the most consistent best match. First, I used the following code to select the best matches per species and annotate them.

```
best.blast<-do.call(rbind,lapply(unique(blast.dat$gene),function(gene,blast.dat){
  dat<-blast.dat[blast.dat$gene==gene,]
  best.matches<-data.frame(stringsAsFactors=FALSE)
    for(spp in unique(dat$query_spp)){
      df<-dat[dat$query_spp==spp,]
      bests<-unique(rbind(min_evalue=df[which.min(df$evalue),],
                  max_length=df[which.max(df$length),],
                  min_gapsno=df[which.min(df$gaps),]))
      best.matches<-rbind(best.matches,bests)
    }
  return(best.matches)
},blast.dat=blast.dat))
write.csv(best.blast,"./pigmentation-evolution/best_blast_ssc.csv")
```

I'll use the scovelli gene annotations to discover the 'full' gene sequences matched by the blast results. Essentially, I'm merging the gff annotations with my best targeted blast results. To speed up this comparison, I wrote an Rcpp function. This analysis used ssc_2016_12_20_chromlevel.gff as the gff file.

```
#read in the gene locations
gff.name<-list.files(path = "../../scovelli_genome/",pattern="ssc_\\d+.*_chromlevel.gff")
if(length(grep("gz",gff.name))>0){
   gff<-read.delim(gzfile(paste("../../scovelli_genome/",gff.name,sep="")),header=F)
} else{
   gff<-read.delim(paste("../../scovelli_genome/",gff.name,sep=""),header=F)
}
colnames(gff)<-c("seqname","source","feature","start","end","score","strand","frame","attribute")
```

```
Rcpp::sourceCpp('../src/annotateBlast.cpp') #use my Rcpp function

blastIDs<-blastAnnotate(best.blast,gff)

best.blast$IDs<-blastIDs
#extract just the gene names to compare to the expression data
best.blast$SSCG<-gsub("^.*ID=(SSCG\\d+);.*$","\\1",blastIDs)
write.csv(best.blast,"./pigmentation-evolution/annotated_pigmentation_best_blast_ssc.csv",
          quote = FALSE,row.names=FALSE)
```

Now I can go gene by gene to pull out all the best matching *S. scovelli* gene names. We can take a look at the number of best-match locations in the scovelli genome and see that most of the ones with a single match are for genes that were only found in a single species:

```
best.blast<-read.csv("./pigmentation-evolution/annotated_pigmentation_best_blast_ssc.csv")
blast.summary<-data.frame(Gene=levels(as.factor(best.blast$gene)),
                          Number_of_SSCG=unlist(lapply(
                            tapply(best.blast$SSCG,best.blast$gene,unique),length)),
                          Number_of_species_matches=unlist(lapply(
                            tapply(best.blast$query_spp,best.blast$gene,unique),length)))
rownames(blast.summary)<-NULL
pander(blast.summary)
```

Gene	Number_of_SSCG	Number_of_species_matches
agrp	1	3
atic	3	3
bnc2	3	6
crybg1a	2	1
csf1ra	2	4
cx39	1	1
dct	1	3
ece2b	1	5
ednrba	1	5
erbb3b	4	6
foxd3	3	7
gart	1	2
gch1	1	4
gja5b	3	2
igsf11	2	6
kcnj13	1	1
kita	1	4
kitlga	1	1
ltk	2	4
mc1r	1	3
mc2r	1	3
mc3r	1	1
mc4r	1	4
mc5ra	2	3
mitfa	4	4
mpv17	1	1
oca2	1	1
paics	1	5
pax7a	2	5
pnp4a	1	1
pomca	1	1
pomcb	1	1
pts	1	1
slc24a5	2	5
slc2a11b	1	1
slc2a15b	1	4
slc45a2	1	4
sox10	4	5
sox5	1	7
tfec	2	3
tjp1a	2	8
tuba8l3	5	5
tyr	1	2
tyrp1b	2	4
xdh	1	2

To identify the information I want (the most likely gene region in the *S. scovelli* genome), I went through these matches by eye. When I was confident in a match, I added the annotation to the candidates.csv file. In some cases, it looked pretty clear that there are two copies of the gene (for example tuba8l3), and with those I chose the gene that matched the *D. rerio* genes, since I'll be using *D. rerio* for KEGG/GO annotations later. Here are the matches:

```
candidates<-read.csv("./pigmentation-evolution/candidates.csv")
pander(candidates[,c("zfin_id_symbol","sscovelli_name")])
```

zfin_id_symbol	sscovelli_name
agrp	SSCG00000004402
agrp	
asip1	
asip2b	
atic	SSCG00000013410
bnc2	SSCG00000019363
crybg1a	
crybg1b	
csf1ra	
cx39.4	
dct	SSCG00000001931
ece2a	
ece2b	SSCG00000001941
ednrba	SSCG00000007649
erbb3b	
foxd3	SSCG00000004645
gart	SSCG00000008168
gch1	SSCG00000012076
gja5b	
igsf11	SSCG00000009593
kcnj13	SSCG00000012942
kita	SSCG00000008311
kitlga	SSCG00000009155
ltk	SSCG00000015167

mc1r	LG4:9005515-9006408
mc2r	SSCG00000017199
mc3r	
mc4r	SSCG00000012642
mc5ra	SSCG00000017194
mc5ra	SSCG00000017194
mitfa	
mpv17	
oca2	
paics	SSCG00000008313
pax7a	SSCG00000010049
pnp4a	
pomca	SSCG00000017691
pomcb	SSCG00000011827
pts	
slc24a5	SSCG00000013437
slc2a11b	
slc2a15b	SSCG00000008460
slc45a2	SSCG00000006320
sox10	SSCG00000020382
sox5	LG17:3731810-3731650
tfec	SSCG00000016281
tjp1a	SSCG00000005955
tuba8l3	SSCG00000019971
tyr	SSCG00000005171
tyrp1a	
tyrp1b	SSCG00000010139

```
  xdh             SSCG00000017674
```

The ones with `NA` either did not have a clear definitive match when looking across multiple species or had no matches at all. Some of them had clear matches to genomic regions that either had multiple gene names or no gene annotations – those are designated by the scaffold they're on and the relevant bp (those scaffolds are generally anchored to chromosomes).

Now I'm going to check the ones with scaffolds:bp instead of a gene ID to see if there are any gene annotations within the region

```
scaffs<-candidates$sscovelli_name[grep("LG",candidates$sscovelli_name)]
startbp<-as.numeric(gsub("LG\\d+:(\\d+)-(\\d+)","\\1",scaffs))
stopbp<-as.numeric(gsub("LG\\d+:(\\d+)-(\\d+)","\\2",scaffs))
scaffs<-gsub("(LG\\d+):(\\d+)-(\\d+)","\\1",scaffs)

ssgs<-lapply(1:length(scaffs),function(n, scaffs,startbp,stopbp,gff){
  this_scaff<-gff[gff$seqname%in%scaffs[n],]
  browser()
  if(nrow(this_scaff)==0){ #then the scaff ID came from attribute not seqname
    this_scaff<-gff[grep(paste0(scaffs[n],"$"),gff$attribute),]
    this_scaff<-gff[gff$seqname%in%this_scaff$seqname,]
  }
  # if the candidate region INCLUDES a gene
  cds<-this_scaff[this_scaff$feature %in% c("gene","CDS","exon","mRNA") &
                    this_scaff$start >= startbp[n] & this_scaff$end <= stopbp[n], ]
  if(nrow(cds)==0){
    # if the candidate region IS INCLUDED IN a gene
    cds<-this_scaff[this_scaff$feature %in% c("gene","CDS","exon","mRNA") &
                      this_scaff$start <= startbp[n] & this_scaff$end >= stopbp[n], ]
  }
  if(nrow(cds)==0){
    return(NA)
  }else{
    sscg<-paste(unique(gsub(".*ID=(SSCG\\d+).*","\\1",cds$attribute)),collapse=";")
    return(sscg)
  }
},scaffs=scaffs,startbp=startbp,stopbp=stopbp,gff=gff)
kable(data.frame(gene=as.character(candidates$zfin_id_symbol[grep("LG",candidates$sscovelli_name)]),
                 ScovelliID=unlist(ssgs)))
```

This did not help me ID any relevant regions, so for now I'll leave these two genes out of the analysis.

Candidate genes in the full skin transcriptome dataset

Drew sequenced transcriptomes of bands in estrogen-exposed males, estrogen-exposed females, control males, and control females. He analysed the transcriptomes for differential expression, which is detailed in a separate file. Here, I am using his differential expression datatable to identify candidate genes.

First I will identify candidate pigmentation genes

```
candidates<-read.csv("./pigmentation-evolution/candidates.csv")
ann<-candidates[candidates$sscovelli_name!="",]
```

```
dat<-read.delim("../data/band_transcriptome/datatable.txt",header=TRUE,sep=" ")
#fix column names
colnames(dat)[9:28]<-paste(colnames(dat)[9:28],"TPM",sep="_")
colnames(dat)[29:48]<-gsub("\\.1","_normCPM",colnames(dat)[29:48])

#add annotations
dat$gene<-unlist(apply(dat,1, function(d,ann){
  this.ann<-ann[ann[,"sscovelli_name"] %in% d[["Annot_Name"]],]
  if(nrow(this.ann)==0){
    match<-NA
  }else{
    match<-this.ann$zfin_id_symbol
  }
  return(unique(as.character(match)))
},ann=ann))
```

Now I've matched the scovelli gene annotations to the expression results, so I'll re-save the data (and read it in using a different name)

```
write.csv(dat,"./pigmentation-evolution/pigmentation_blast_matched_to_band_genes.csv",
          quote=FALSE,row.names = FALSE)
```

```
expr.blast<-read.csv("./pigmentation-evolution/pigmentation_blast_matched_to_band_genes.csv")
```

26 of the 12693 genes in Drew's transcriptome dataset match 26 of the 1 annotated pigmentation genes. These genes are: xdh, pomca, gart, pomcb, sox10, tfec, tyr, igsf11, ltk, kitlga, pax7a, mc5ra, gch1, tjp1a, slc24a5, atic, slc2a15b, tyrp1b, ednrba, tuba8l3, dct, foxd3, paics, kita, bnc2, kcnj13.

What about expression levels of the putative pigmentation genes?

Drew's data conveniently includes the differential expression comparisons for these annotated pigmentation genes found in the transcriptome. But first, let's look at the expression of just these genes visually.

```
#sort by gene
label.colors<-c(EF="#d01c8b",CF="#f1b6da",CM="#b8e186",EM="#4dac26")
ann.expr<-expr.blast[!is.na(expr.blast$gene),]
ann.expr<-ann.expr[order(ann.expr$gene),]
rownames(ann.expr)<-NULL
```

```
png("../figs/estrogen-bands/estrogen_nodendro_HM.png",height=7,width = 7,res=300,units="in")
h<-heatmap(as.matrix(ann.expr[order(ann.expr$gene,decreasing=TRUE),
                              grep("TPM",colnames(ann.expr))]),
           Rowv=NA,Colv=NA,labRow = ann.expr$gene[order(ann.expr$gene,decreasing=TRUE)],
           ColSideColors = c(rep(label.colors["CM"],5),rep(label.colors["EM"],5),
                             rep(label.colors["CF"],5),rep(label.colors["EF"],5)))
dev.off()
```

```
## png
##   2
```

```
png("../figs/estrogen-bands/estrogen_dendro_HM.png",height=7,width = 7,res=300,units="in")
h<-heatmap(as.matrix(ann.expr[order(ann.expr$gene,decreasing=TRUE),
                              grep("TPM",colnames(ann.expr))]),
           Rowv=NA,labRow = ann.expr$gene[order(ann.expr$gene,decreasing=TRUE)],
           ColSideColors = c(rep(label.colors["CM"],5),rep(label.colors["EM"],5),
```

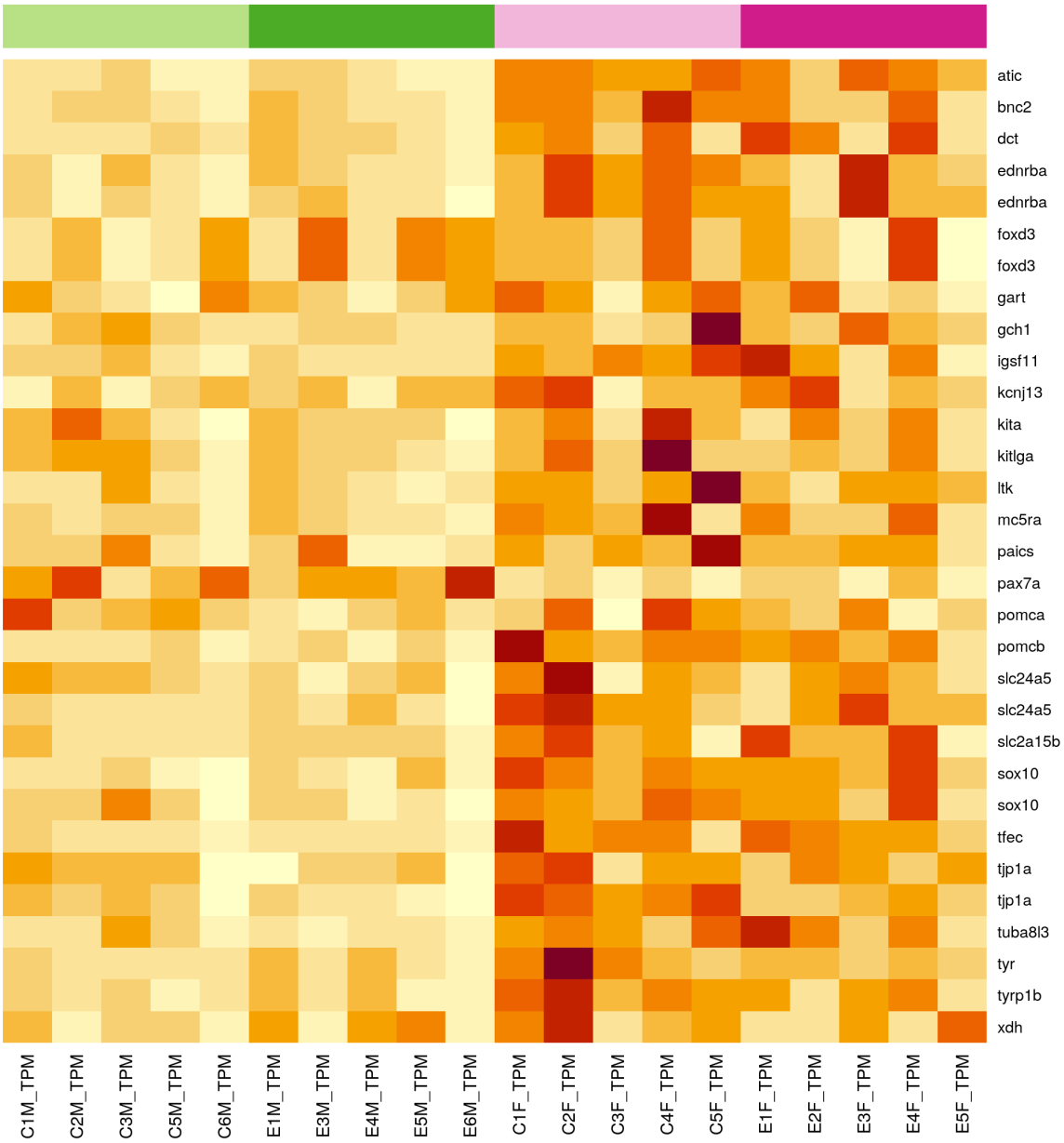

Figure 6: Heatmap without dendrogram. The colors at the top represent the groups, where light green are control males, dark green are estrogen-exposed males, light purple are control females, and dark purple are estrogen-exposed females.

```
                              rep(label.colors["CF"],5),rep(label.colors["EF"],5)))
dev.off()
```

```
## png
##   2
```

For comparison, we can look at a heatmap of all of the genes

```
png("../figs/estrogen-bands/estrogen_allgenes_HM.png",height=7,width = 7,res=300,units="in")
h<-heatmap(as.matrix(expr.blast[order(expr.blast$gene,decreasing=TRUE),
                                grep("TPM",colnames(expr.blast))]),
           Rowv=NA,labRow = expr.blast$gene[order(expr.blast$gene,decreasing=TRUE)],
           ColSideColors = c(rep(label.colors["CM"],5),rep(label.colors["EM"],5),
                             rep(label.colors["CF"],5),rep(label.colors["EF"],5)))
dev.off()
```

```
## png
##   2
```

Now that I've visualized these, I'll focus in on the genes that have significant FDR values:

```
sig.eb<-expr.blast[expr.blast$CFvEF_FDR <=0.05 | expr.blast$CMvCF_FDR <= 0.05 |
                     expr.blast$CMvEF_FDR <= 0.05 | expr.blast$CMvEM_FDR <=0.05,]
```

```
#number sig in each
sig.counts<-data.frame(Comparison=c("Control Female - Exposed Female",
                                    "Control Male - Exposed Male",
                                    "Control Male - Exposed Female",
                                    "Control Male - Control Female"),
                       Number_Significant=c(length(which(expr.blast$CFvEF_FDR <=0.05)),
                                            length(which(expr.blast$CMvEM_FDR <=0.05)),
                                            length(which(expr.blast$CMvEF_FDR <=0.05)),
                                            length(which(expr.blast$CMvCM_FDR <=0.05))))

pander(sig.counts)
```

Comparison	Number_Significant
Control Female - Exposed Female	2
Control Male - Exposed Male	30
Control Male - Exposed Female	1293
Control Male - Control Female	0

These 1560 genes are all significant in one of the male-female comparisons (either control male vs control female or control male vs estrogen female) – suggesting that these may be sex-specific rather than estrogen responsive. To some extent this fits with Drew's overall results, which are that the major pattern of differential expression is not hugely estrogen responsive.

However, 3 differentially expressed genes are candidate genes: *atic*, *pax7a*, and *tfec*. These genes have the following significance values:

```
pander(cbind(sig.eb[!is.na(sig.eb$gene),grep("FDR",colnames(sig.eb))],
             gene=sig.eb[!is.na(sig.eb$gene),"gene"]))
```

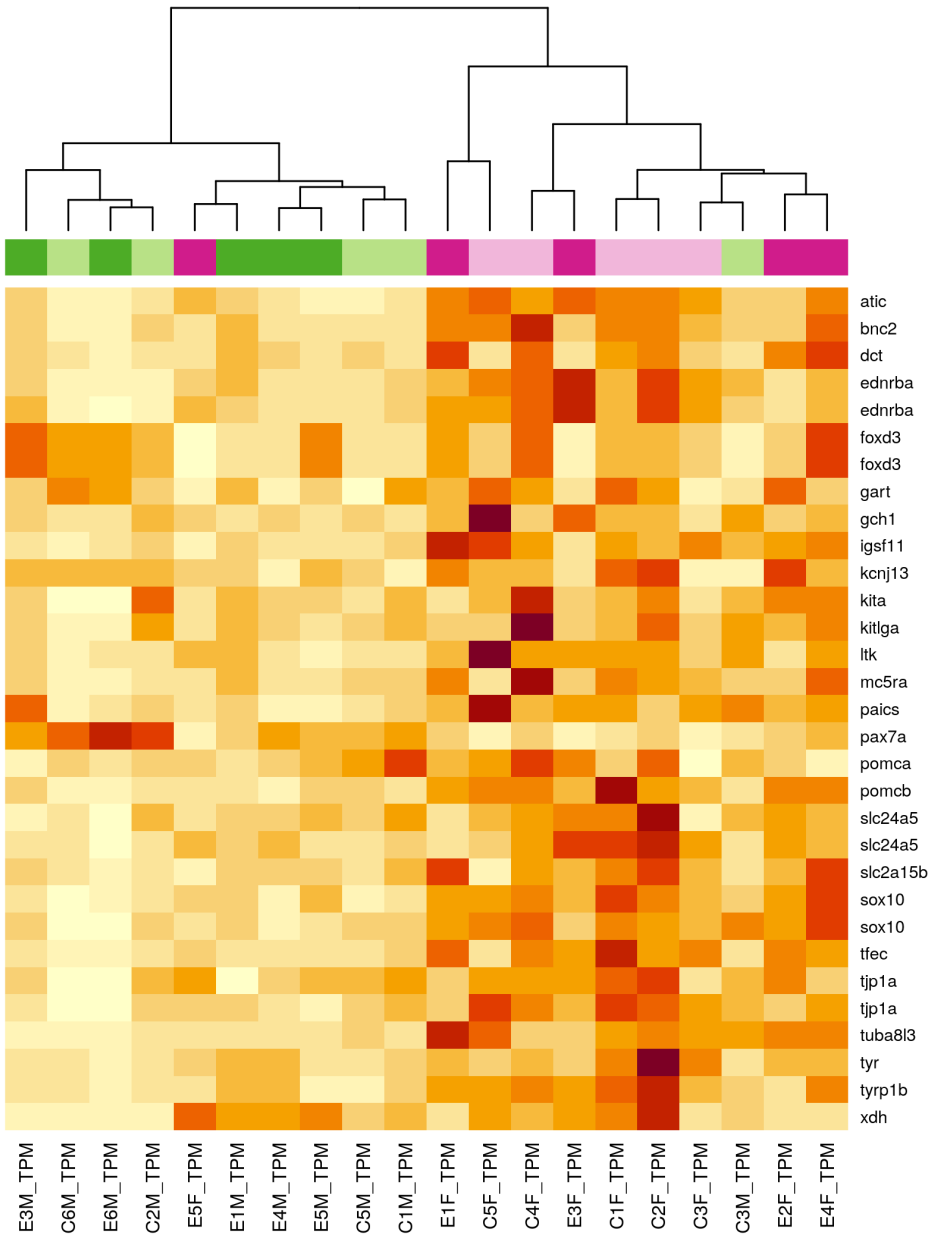

Figure 7: Heatmap with dendrogram. The colors at the top represent the groups, where light green are control males, dark green are estrogen-exposed males, light purple are control females, and dark purple are estrogen-exposed females.

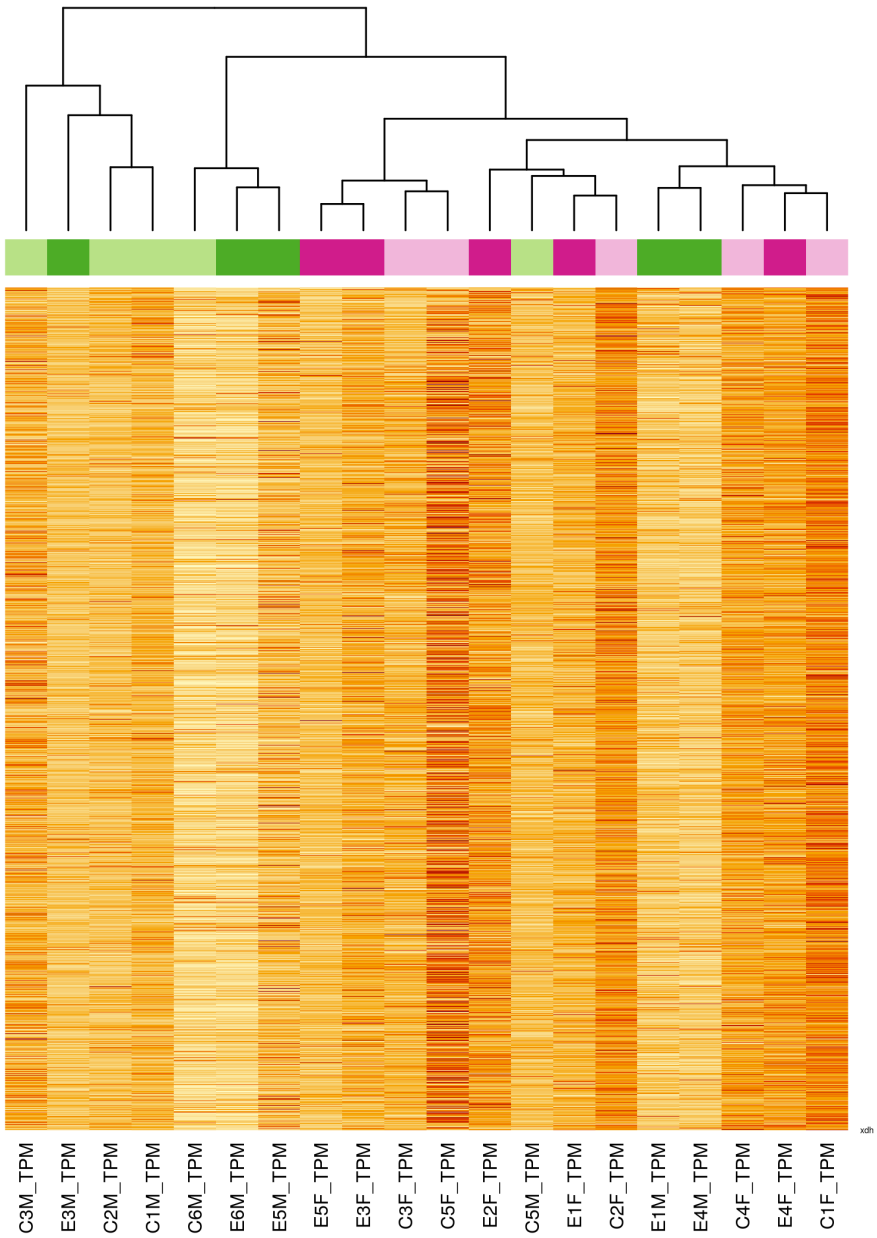

Figure 8: Heatmap of all genes. The colors at the top represent the groups, where light green are control males, dark green are estrogen-exposed males, light purple are control females, and dark purple are estrogen-exposed females.

Table 5: Table continues below

	CMvEM_FDR	CMvCF_FDR	CMvEF_FDR	CFvEF_FDR	EMvCF_FDR
6514	1	0.01828	0.02958	1	0.05451
8692	1	0.06494	0.01322	1	0.003033
11848	0.9524	0.008883	0.02242	1	0.1944

	EMvEF_FDR	gene
6514	0.04015	tfec
8692	0.02017	pax7a
11848	0.1235	atic

Both appear to have constitutive sex-biased gene expression, and this sex bias may be more extreme when females are exposed to estrogen (do we have comparisons of exposed males to control females? that may lend some insight into this result).

Two of these genes have both been implicated in zebrafish studies to be involved in iridophore proliferation. *atic* is involved in guanine synthesis and was found by (Higdon 2013) to be upregulated in iridophores relative to melanophores. *tfec* was even more directly implicated in iridophore specification by (Petratou 2018): *tfec* was observed to have positive feedback with *ltk* that drove the specification of iridophores. The third gene, *pax7a*, has been implicated in medaka as being involved in the differentitation of xanthophores from leucophores (Kimura et al. 2014).

Now we'll calculate the mean log fold changes to report in the paper:

```
sig.genes<-cbind(sig.eb[!is.na(sig.eb$gene),],
             gene=sig.eb[!is.na(sig.eb$gene),"gene"])
means<-sig.genes[,grep("logFC",colnames(sig.genes))]
rownames(means)<-sig.genes$gene
pander(means)
```

Table 7: Table continues below

	CMvEM_logFC	CMvCF_logFC	CMvEF_logFC	CFvEF_logFC
tfec	0.177	1.296	1.175	0.1213
pax7a	0.2414	-1.509	-1.843	0.334
atic	0.4501	1.235	1.076	0.1584

	EMvCF_logFC	EMvEF_logFC
tfec	0.9982	1.119
pax7a	-2.084	-1.75
atic	0.6262	0.7845

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