How the Devil Ray Got Its Horns: The Evolution and Development of Cephalic Lobes in Myliobatid Stingrays (Batoidea: Myliobatidae)

John D. Swenson[1]*, Jeff Klomp[2], Robert A. Fisher[3] and Karen D. Crow[1]

[1] Department of Biology, San Francisco State University, San Francisco, CA, United States, [2] Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, United States, [3] Virginia Institute of Marine Science, College of William and Mary, Williamsburg, VA, United States

Manta rays and their relatives of the family Myliobatidae have pectoral fins that have been modified for underwater flight, as well as a pair of fleshy projections at the anterior of the body called cephalic lobes, which are specialized for feeding. As a unique trait with a dedicated function, cephalic lobes offer an excellent opportunity to elucidate the processes by which diverse body plans and features evolve. To shed light on the morphological development and genetic underpinnings of cephalic lobes, we examined paired fin development in cownose rays, which represent the sister taxon to manta rays in the genus *Mobula*. We find that cephalic lobes develop as anterior pectoral fin domains and lack independent posterior patterning by *5′ HoxD* genes and *Shh*, indicating that cephalic lobes are not independent appendages but rather are modified pectoral fin domains. In addition, by leveraging interspecies comparative transcriptomics and domain-specific RNA-sequencing, we identify shared expression of anterior patterning genes, including *Alx1*, *Alx4*, *Pax9*, *Hoxa13*, *Hoxa2,* and *Hoxd4*, in the pectoral fins of cownose ray (*Rhinoptera bonasus*) and little skate (*Leucoraja erinacea*), providing evidence supporting homology between the cephalic lobes of myliobatids and the anterior pectoral fins of skates. We also suggest candidate genes that may be involved in development of myliobatid-specific features, including *Omd*, which is likely associated with development of thick anterior pectoral fin radials of myliobatids, and *Dkk1*, which may inhibit tissue outgrowth at the posterior boundary of the developing cephalic lobes. Finally, we observe that cephalic lobes share a surprising number of developmental similarities with another paired fin modification: the claspers of male cartilaginous fishes, including enrichment of *Hand2*, *Hoxa13*, and androgen receptor. These results suggest that cephalic lobes may have evolved by co-opting developmental pathways that specify novel domains in paired fins. Taken together, these data on morphological development and comparative gene expression patterns illustrate how distinct body plans and seemingly novel features can arise via subtle changes to existing developmental pathways.

Keywords: manta ray, batoid, myliobatid, cephalic lobes, RNA-seq, gene expression, fin development, comparative transcriptomics

INTRODUCTION

Manta rays (also known as devil rays) and their relatives of the family Myliobatidae exhibit modified body plan features that arose in association with the evolution of oscillatory swimming and invasion of the pelagic environment (Rosenberger, 2001; Schaefer and Summers, 2005; Mulvany and Motta, 2013; Hall et al., 2018). While myliobatids share classic components of the batoid body plan, including dorsoventral compression and pectoral fins that expand anteriorly and fuse to the head, they also exhibit derived morphological modifications that are unique to the family, such as anterior projections called "cephalic lobes" that are used to locate and manipulate prey during feeding (Sasko et al., 2006; Mulvany and Motta, 2014), loss of anterior-posterior disc symmetry typical of most skates and rays (Hall et al., 2018) and laterally extended pectoral fins that arose in association with oscillatory swimming (Fontanella et al., 2013; Franklin et al., 2014; Martinez et al., 2016). While most batoids use their broad disc or diamond-shaped pectoral fins for both swimming and feeding, the myliobatids exhibit functional separation of swimming and feeding behaviors into the pectoral fins and cephalic lobes, respectively, which have been optimized for those specific tasks (Mulvany and Motta, 2013; Hall et al., 2018).

The separation of structures dedicated to feeding and locomotion appears to be associated with a large-scale remodeling of the myliobatid body plan, particularly in the pectoral fins. For example, the skeletal elements in the anterior pectoral fins of myliobatids fuse at the interradial joints to form the *compagibus laminam*, a plate of skeletal elements that likely promotes lift during steady swimming (Schaefer and Summers, 2005; Hall et al., 2018). In contrast, fin rays in the posterior pectoral fins of myliobatids are thinner and more numerous than those of non-myliobatids, which likely contributes to increased maneuverability (Hall et al., 2018). In addition, myliobatid pectoral fins are laterally expanded, resulting in a high aspect ratio that reduces drag while increasing lift and thrust generation (Fontanella et al., 2013; Franklin et al., 2014), the center of mass is shifted anteriorly, which stabilizes the body during steady swimming (Fontanella et al., 2013), and the pectoral fins are stiffened by calcification patterns in the radials that redistribute load-bearing elements and increase the power of high amplitude pectoral strokes (Schaefer and Summers, 2005). Together, these modifications are associated with the evolution of oscillatory swimming, or underwater flight, the primary mode of locomotion in myliobatids.

Though these morphological adaptations promote efficient long-range swimming in the pelagic environment, they also likely represent constraints on feeding, as stiffened pectoral fins are not well-suited for the prey capture strategies observed in other batoids, such as tenting prey against the substrate (Wilga et al., 2012; Mulvany and Motta, 2014), which requires flexibility at the anterior of the pectoral fins. As such, myliobatids have evolved cephalic lobes, fleshy anterior appendages that are used primarily for feeding and exhibit a degree of flexibility that is comparable to the anterior pectoral fins of other batoids (Mulvany and Motta, 2013).

Cephalic lobes (sometimes referred to as cephalic fins) exhibit a variety of shapes, sizes, and functions (Nishida, 1990; Miyake et al., 1992; Mulvany and Motta, 2013). The most widely recognized example of cephalic lobes occurs in manta rays of the genus *Mobula* (Aschliman, 2014; Poortvliet et al., 2015; White et al., 2017), in which the elongated cephalic lobes unfurl to funnel plankton into the mouth when feeding (Notarbartolo-Di-Sciara, 1987; Notarbartolo-di-Sciara and Hillyer, 1989; Ari and Correia, 2008). In cownose rays (*Rhinoptera*), the flexible paired cephalic lobes are fused at the midline and used to trap prey against the substrate, analogous to the tenting behavior performed by the anterior pectoral fins of other batoids (Sasko et al., 2006; Fisher et al., 2011; Mulvany and Motta, 2014), and in bat rays (*Myliobatis*) and eagle rays (*Aetobatus* and *Aetomylaeus*), the cephalic lobes are fused into a single structure that is used like a shovel to unearth benthic prey (Mulvany and Motta, 2013). Cephalic lobes are covered in electrosensory pores that are used for locating hidden prey in all myliobatid genera except *Mobula*, which lack pores (Jordan, 2008; Mulvany and Motta, 2013; Bedore et al., 2014). By adopting key functions associated with feeding, such as prey detection and capture, cephalic lobes may have released the pectoral fins from constraints associated with feeding, thereby facilitating the evolution of oscillatory swimming in the Myliobatidae and subsequent invasion of the pelagic environment.

Despite their significant role in myliobatid ecology and evolution, the mechanisms underlying the evolution and development of cephalic lobes remain a mystery. Myliobatid taxa with paired cephalic lobes have been referred to as the only vertebrates with three pairs of functional appendages (Nelson et al., 2016). If cephalic lobes are independent appendages, then we would expect them to develop separately from the pectoral fins and to exhibit independent canonical expression of genes that pattern paired appendages, such as posterior patterning by 5′ *HoxD* genes (see Kmita et al., 2005; Freitas et al., 2007, 2012) and *Shh* expression (see Krauss et al., 1993; Riddle et al., 1993; Chang et al., 1994; Dahn et al., 2007). However, most examples of novel morphological traits arise from existing or duplicated structures that are modified to the extent that homology becomes obscured. As such, cephalic lobes may have evolved as modifications to the anterior pectoral fins, in which case we would expect them to develop in association with anterior pectoral fins, lack independent posterior canonical fin/limb patterning and to share homologous gene expression patterns with the anterior pectoral fins of other batoids that lack cephalic lobes. Cephalic lobes, therefore, represent an excellent opportunity to inform the mechanisms by which unique morphological features evolve in association with novel functions and ecological roles.

Because manta rays (genus *Mobula*) are exceptionally challenging to sample, and many mobulids are listed as vulnerable or endangered (Couturier et al., 2012), we focused our efforts on a representative of their sister taxon: cownose rays of the genus *Rhinoptera*, which exhibit a similar body plan, including paired cephalic lobes. To elucidate the morphological and molecular evolution and development of myliobatid cephalic lobes, we sampled embryos of the cownose ray, *Rhinoptera bonasus*, during outgrowth and patterning of the pectoral fins

and characterized their developmental progression. To evaluate homology between myliobatid cephalic lobes and the anterior pectoral fins of a batoid that lacks cephalic lobes, we sequenced RNA from developing cownose ray cephalic lobes and pectoral fins for comparison with gene expression patterns in developing pectoral fins of the little skate (Nakamura et al., 2015) using similar methodology. Using this dual-pronged approach, we addressed the following specific questions:

1) Do cephalic lobes *develop* as independent paired appendages or do they develop as modified anterior pectoral fins?
2) Are myliobatid cephalic lobes and rajid anterior pectoral fins *patterned* by shared developmental gene expression that would support homology?
3) Can we identify distinct developmental processes and gene expression patterns associated with myliobatid-specific features, including cephalic lobes?

To our knowledge, this is the first study to document fin development in the myliobatid lineage. Our results indicate that cephalic lobes do not develop as independent appendages, but rather as modifications of the anterior pectoral fins which share homology with the anterior pectoral fins of skates. We also offer a description of the processes underlying development of myliobatid pectoral fins and cephalic lobes, which includes distinct phases of anterior expansion followed by fusion to the pharyngeal basket, head, and rostrum. We further identify gene expression patterns likely associated with myliobatid-specific pectoral fin modifications, including specification of cephalic lobes, the *compagibus laminam,* and a myliobatid-specific developmental feature we call the "notch." Finally, we found enrichment of shared genes during development of both claspers and cephalic lobes which also share several morphological features, suggesting a redeployment of pathways that specify development of these paired fin modifications.

METHODS

Sample Collection and Developmental Staging

It is intractable to sample myliobatid embryos at specific developmental stages, as there is no published developmental timeline for any myliobatid taxon and no appropriately comparable model taxa have been characterized. Therefore, we conducted a pilot study to delineate the window of pectoral fin development in cownose rays from a wild population in Chesapeake Bay. Then, working with the bycatch of commercial fishermen, we sampled during July and August of 2016 and 2017. Embryos used for RNA-sequencing were preserved in RNALater, incubated at 4°C for 24–72 h, and then stored at −20°C until dissection and RNA extraction. Samples used for staging were fixed in 4% paraformaldehyde for 48 h before being transferred to methanol and stored at −80°C. Embryos were staged in an RNase-free environment, grouped by characters that we observed to be consistently associated with development of the pectoral fins (**Table 1**), and assigned a numerical index representing these characters (**Table 2**).

One goal of this study was to evaluate homology between the anterior pectoral fins of skates and the cephalic lobes of myliobatids using comparative transcriptomics. Because developmental-genetic programs can shift in space and time, it is important to examine similar developmental stages in each taxon. Therefore, morphological characters that were shared between developing cownose ray and winter skate (*Leucoraja ocellata*, following Maxwell et al., 2008) pectoral fins were identified. These were used to determine the most comparable stages of development in the cownose ray, *Rhinoptera bonasus* (*R. bonasus*), and little skate, *Leucoraja erinacea* (*L. erinacea*) (**Table S1**) for gene expression analysis.

Of all stages collected, the stage three *R. bonasus* embryos we sampled for RNA-sequencing represented the most biological replicates (six), and therefore gave us the greatest power to detect differentially expressed genes (DEGs). In addition, the pectoral fins undergo significant developmental processes during this stage, including anterior expansion, similar to stage 31 *L. erinacea* embryos sequenced by Nakamura et al. (2015). Due to the enhanced power to detect DEGs, the active developmental processes (including anterior expansion of the pectoral fins), and the availability of comparable data in *L. erinacea*, we focused our molecular analyses on stage three of *R. bonasus* development.

RNA Sequencing of Distinct Pectoral Fin Domains

Nakamura et al. (2015) sequenced RNA from three equal-sized pectoral fin domains of developing embryos of *L. erinacea*, a representative batoid from the skate family (Rajidae), which diverged from all other batoids approximately 180 million years ago (Aschliman et al., 2012). We used these data for comparison to address the question of cephalic lobe/pectoral fin homology. To replicate the methods of Nakamura et al. (2015), we cut the left pectoral fin of stage three *R. bonasus* embryos (which includes the cephalic lobe) in thirds. We then added a layer of resolution to our dissections by bisecting each third of the pectoral fin again. The cephalic lobe comprised a distinct domain when the fin was split in this way (**Figure 1**). Further, when we included RNA-sequencing reads from adjacent dissections, our data represented three evenly-sized pectoral fin domains (following Nakamura et al., 2015; see **Figure 1**) with which to evaluate homology with the three domains previously examined in *L. erinacea*.

Following dissection, RNA was extracted from the six *R. bonasus* pectoral fin domains, as well as two additional fin domains, including pelvic fin. We extracted RNA using Trizol and followed with column-based purification using the PureLink RNA mini kit (Invitrogen) and a subsequent DNase I digestion. Purity was assessed using a NanoDrop with 260/280 ratios registering between 2.0 and 2.5. When there was residual guanidine salt, as indicated by a 260/230 ratio < 1.5, a SPRI bead purification was conducted and the eluate was reanalyzed to confirm that the final 260/230 ratio was greater than 1.5.

Sample aliquots were diluted and RNA integrity numbers (RIN) were evaluated using an RNA Pico chip on an Agilent BioAnalyzer. All samples exhibited RIN scores >8. Total RNA from each sample was quantified using a Qubit and 1 μg of

TABLE 1 | Developmental index parameters based on characters associated with development of cownose ray pectoral fins.

Pectoral fin fusion	Index	Cephalic lobe (CL) fusion	Index	Notch position	Index	Gill arch ectoderm fusion	Index
None	0	None; CL hooked	0	Posterior to 2nd gill arch	0	None	0
To 1st gill arch	1	None; CL partially unfurled	1	Between 2nd and 4th gill arch	1	Partial	1
To 2nd gill arch	2	None; CL fully unfurled	2	5th gill arch	2	Complete	2
To 3rd gill arch	3	Partial; CL only fused to CFT at base	3	Anterior to 5th gill arch, but posterior to mouth	3		
To 4th gill arch	4	Partial; CFT partially covers CL distally	4	Level with mouth	4		
To 5th gill arch	5	Complete; CFT and CL still distinguishable	5	Anterior to mouth	5		
All gill arches plus shoulder	6	Complete; CFT and CL indistinguishable	6				

These indices were added to the disc width to body length (DW:BL) ratio to create a staging index for early pectoral fin development in myliobatids (see **Table 2**).

TABLE 2 | Characters evaluated in developmental index and range of scores for each stage (See **Table 1**).

Stage	Replicates	DW:BL ratio	Pectoral fin fusion	Cephalic lobe fusion	Notch position	Gill arch ectoderm fusion	Total Index
1	5	0.4–0.6	0–1	0	0–1	0	0.4–2.6
2	5	0.6–0.7	1–3	0–1	1	0	3.6–5.7
3	6	0.7–0.9	4–6	2	1–2	0	8.7–10.9
4	8	0.7–0.9	6	3	3	1	13.7–13.9
5	7	0.9–1.2	6	4	4	1–2	15.0–17.1
6	3	1.2–1.4	6	5	4	2	18.2–18.4
7	4	1.2–1.7	6	5–6	4–5	2	19.2–21.7

RNA from each sample was used for Poly(A) mRNA enrichment using NEBNext oligo d(T) magnetic beads. After mRNA isolation and fragmentation, cDNA libraries were constructed using the NEBNext Ultra Directional Library Prep Kit for Illumina and each sample was given a unique barcode. Double-sided size selection was performed using SPRI beads targeting 400 bp fragments. Fragment size, concentration, and library purity were verified using a High Sensitivity DNA chip on a BioAnalyzer. In total, 48 libraries representing eight distinct fin domains with six biological replicates were normalized, pooled, and sequenced on an Illumina HiSeq 4000 using 100 bp paired end (PE) reads. Final library quality assessment and sequencing were conducted at the University of California, Berkeley; all other labwork occurred at San Francisco State University.

The cownose ray is a non-model organism, and the closest taxon for which a published genome exists is the elephant shark (Venkatesh et al., 2014), which diverged from the cownose ray and other elasmobranchs about 420 million years ago (Inoue et al., 2010). When sequence divergence is >15%, *de novo* transcriptome assemblies are recommended over reference-based mapping approaches (Vijay et al., 2013). To aid the *de novo* assembly process, we sequenced additional cephalic lobe, clasper, and pectoral fin domains on an Illumina MiSeq with longer reads (250 and 300 bp PE). The longer reads facilitated *de novo* transcriptome assembly by helping to bridge low complexity and redundant regions to which the shorter HiSeq reads could

map. These samples were not included in our DGE analyses because they were prepared and sequenced using distinct kits and methodologies, which may introduce significant biases in expression levels (van Dijk et al., 2014).
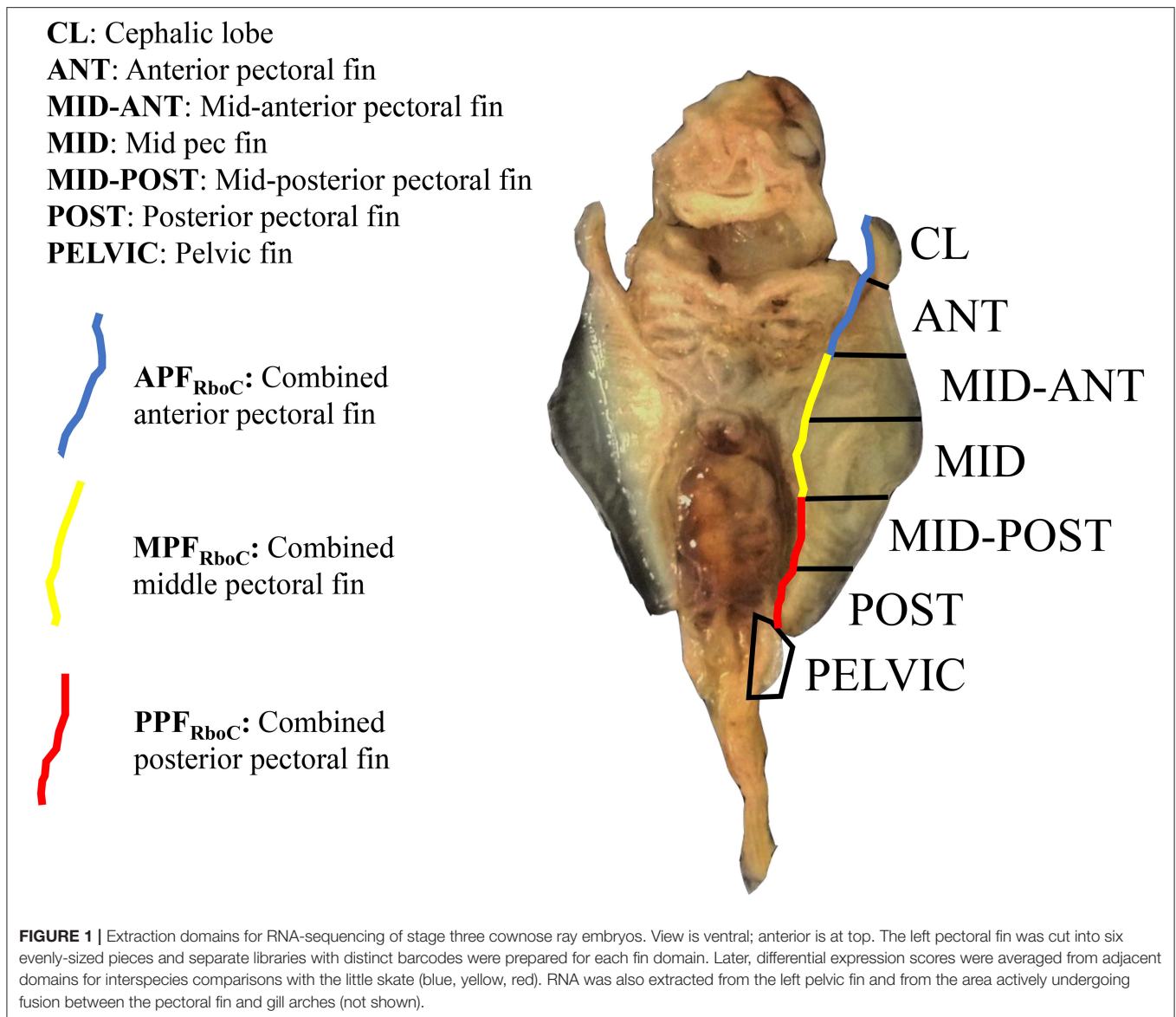
Data Analysis
Data Preprocessing
Adapters and low quality sequences from cownose ray data were trimmed from fastq files using the TrimGalore (Krueger, 2012) wrapper for Cutadapt (Martin, 2011) and FastQC (Andrews, 2010). Overlapping reads were combined with Flash (Magoc and Salzberg, 2011) prior to assembly and orphaned reads were retained. To reduce the impact of genetic variability, data from fin domains of only three individuals were used in the assembly, including: two regions of a stage one embryo (broad anterior pectoral fin region and mid-anterior pectoral fin), one region of a stage seven embryo (clasper) and eight regions of stage three embryos (cephalic lobe, anterior pectoral fin, mid-anterior pectoral fin, mid-pectoral fin, mid-posterior pectoral fin, posterior pectoral fin, zipper region, and pelvic fin). Read counts before and after filtering are reported in **Table S2**.

Transcriptome Assembly and Annotation
Sequencing with both HiSeq (stage 3 samples, 100 bp PE reads) and MiSeq (stage 1 samples, stage 7 sample, 300 bp PE reads) was conducted to help generate full length transcripts. Abyss

CL: Cephalic lobe
ANT: Anterior pectoral fin
MID-ANT: Mid-anterior pectoral fin
MID: Mid pec fin
MID-POST: Mid-posterior pectoral fin
POST: Posterior pectoral fin
PELVIC: Pelvic fin

APF$_{RboC}$: Combined anterior pectoral fin

MPF$_{RboC}$: Combined middle pectoral fin

PPF$_{RboC}$: Combined posterior pectoral fin

CL
ANT
MID-ANT
MID
MID-POST
POST
PELVIC

FIGURE 1 | Extraction domains for RNA-sequencing of stage three cownose ray embryos. View is ventral; anterior is at top. The left pectoral fin was cut into six evenly-sized pieces and separate libraries with distinct barcodes were prepared for each fin domain. Later, differential expression scores were averaged from adjacent domains for interspecies comparisons with the little skate (blue, yellow, red). RNA was also extracted from the left pelvic fin and from the area actively undergoing fusion between the pectoral fin and gill arches (not shown).

(Simpson et al., 2009) was used to assemble contiguous sequences (contigs) from the paired end (PE) and orphaned sequence data with 10 km sizes ranging from 25 to 70 nucleotides. Contigs of 500 nucleotides or greater were summarized according to similarity (e-value < 1e-160) with BLAST+ tools (Camacho et al., 2009) into 63,441 unique contigs.

Using iterative BLAST searches and pairwise alignments, contig sequences with strong e-values (<1e-10) were annotated and used in the final transcriptome. Specifically, initial screening and annotation of contigs was conducted iteratively by best-reciprocal-blast using peptide, coding sequence, and non-coding libraries from *Danio rerio* (GRCz10) with BLAST+ tools and a minimum e-value threshold of 0.1. Each *R. bonasus* contig was paired with *D. rerio* sequences and the lowest average e-value was chosen as the annotation. Therefore, more than one *R. bonasus* contig could be designated with the same *D. rerio* sequence, allowing for potential redundancy in

the *de novo R. bonasus* transcriptome assembly. The longest open reading frames (ORFs) that were unannotated to *D. rerio* sequences were annotated by BLASTp using the RefSeq vertebrate protein database (downloaded 4-4-2017). Remaining unannotated contigs were designated by their top forward BLAST hit in *D. rerio*, resulting in a total transcriptome size of 27,900 contigs. For consistency, the same process was used in generating the *L. erinacea* transcriptome from public stage 31 embryo *L. erinacea* 100 bp PE RNA-seq data (SRA, PRJNA288370, Nakamura et al., 2015), generating 24,006 transcripts.

Annotations of genes highlighted in **Figures 4** and **5** were individually verified using NCBI's BLASTn (Altschul et al., 1990). We examined the top vertebrate hits, including, but not limited to, the elephant shark (*Callorhincus milli*), zebrafish (*Danio rerio*), coelacanth (*Latimeria chalumnae*), and mouse (*Mus musculus*), in addition to the catshark (*Scyliorhinus sp.*) when sequence data were available. BLAST e-values and

accession numbers for the sequences to which the *R. bonasus* and *L. erinacea* transcripts most reliably aligned are reported in **Table S3**. Transcripts that were annotated as a different gene in more than one taxon were excluded from analysis due to potential ambiguity in the annotation. One exception was *Ntrk2*, which had the highest overall BLAST score when aligned with *Bdnf* from the whale shark, *Rhinocodon typus* (*e*-value = 0.00; Accession XM_020520327.1), but otherwise preferentially aligned with *Ntrk2* from the other taxa evaluated (**Table S3**). However, the two genes function in tandem: *Ntrk2* is a receptor that shows a high affinity for binding *Bdnf* (Agerman, 2003) and has been used as an indicator of *Bdnf* activity (Pita-Thomas et al., 2010), so we did not discard this gene. Due to their highly conserved homeodomain (Krumlauf, 1994), we were able to translate and align all of the various Hox genes highlighted in the text and phylogenetically confirm the correct annotation of Hox genes using Geneious 11.1.15 (**Figure S1**).

Alignment and Additional Filtering

R base (Ihaka and Gentleman, 1996) and Bioconductor (Gentleman et al., 2004) software packages were used for alignment of quality filtered PE data and differential expression comparisons. Strand-specific alignment was conducted using the seed-and-vote methodology for uniquely aligned reads, as implemented in the Rsubread package (Liao et al., 2013), to the annotated *R. bonasus* transcriptome. Rsamtools was used for file indexing and manipulation (Morgan et al., 2018). Due to potential biases unrelated to developmental gene expression differences, contig sequences annotated as ribosomal, mitochondrial, predicted, hypothetical, location, or uncharacterized were removed from consideration in differential expression. Moreover, only contigs that were represented by more than 100 reads across the full data set of 48 samples and more than 50 reads in at least one individual were considered reasonable to use for differential expression comparisons such that 11,881 transcripts were considered in the differential expression comparisons. Data from *L. erinacea* and *R. bonasus* were processed similarly, except that in *L. erinacea*, the filter required at least 50 reads across all samples (after removing highest) and at least one sample that had at least 25 reads, which was used to help minimize the effect of outliers in the smaller sample set (*N* = 8). In total, 12,822 *L. erinacea* transcripts were considered in the differential expression comparisons.

Differential Expression

Normalization of read count data for the filtered transcripts was conducted with the trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010). EdgeR (Robinson et al., 2010) was used to conduct quasi-likelihood F-tests between groups of samples and false discovery rate adjusted *p*-values (Benjamini-Hochberg) were used to control for multiple testing.

Interspecies Comparisons

For interspecies comparisons, we included RNA-sequencing reads from adjacent domains in *R. bonasus* (**Figure 1**) to replicate the three evenly-sized pectoral fin domains sequenced by Nakamura et al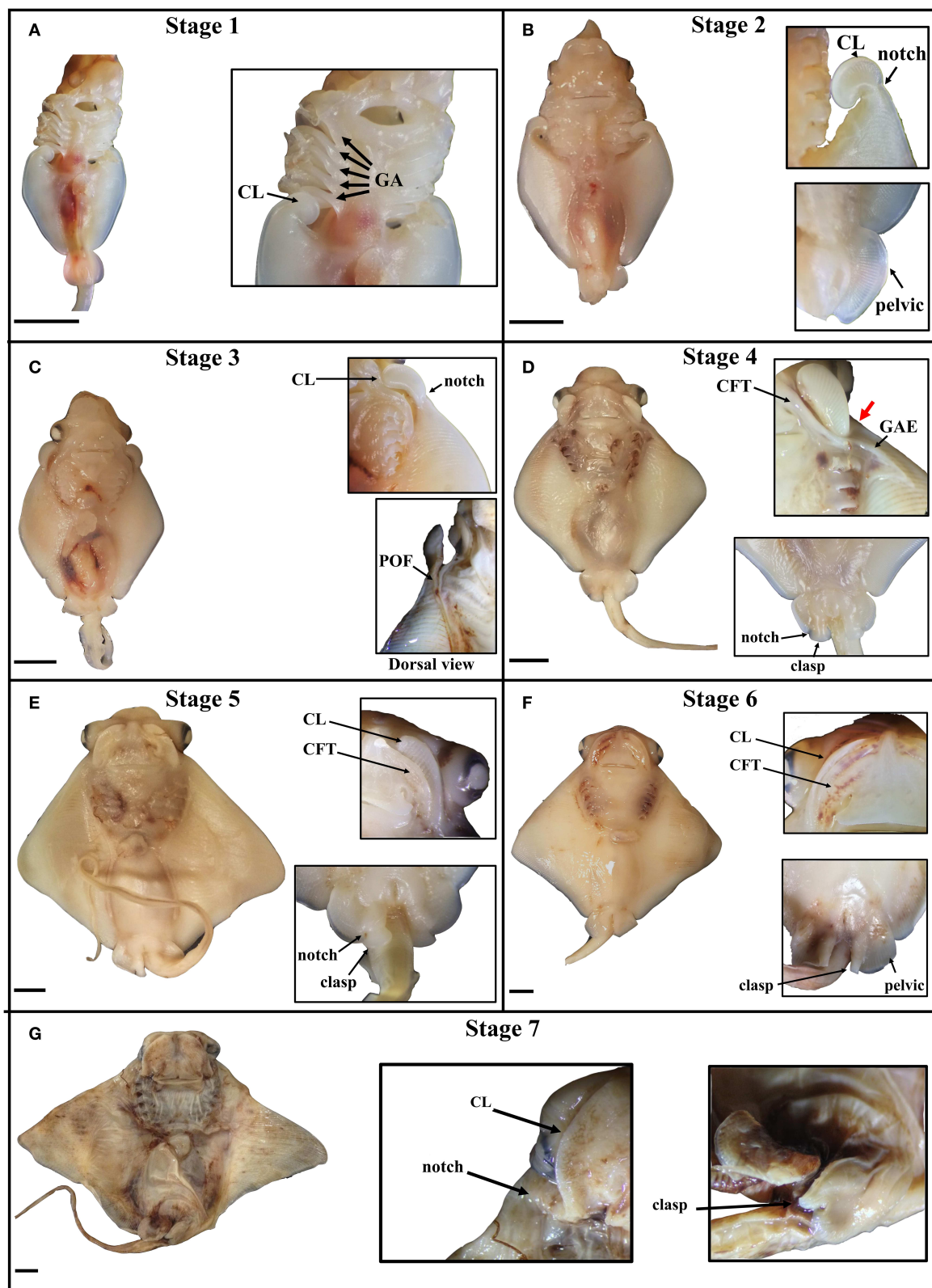. (2015) in *L. erinacea*. We created lists of differentially expressed genes in each species separately and queried the lists of DEGs for genes known to be associated with paired fin development in *L. erinacea* and other non-batoid taxa. This approach accounts for bias associated with library preparation, sequencing, and counting efficiency by comparing ratios of differentially expressed genes among fin domains within each species separately (according to Dunn et al., 2013).

RESULTS

Patterns of Development in Cownose Ray Pectoral Fins

The stages of cownose ray fin development documented here are roughly analogous to stages 28–31 in bamboo sharks and catsharks (Ballard et al., 1993; Onimaru et al., 2018), though we note that sharks lack the anterior expansion of the pectoral fins characteristic of the Batoidea. This anterior expansion appears to occur via similar mechanisms across batoid clades. In *Raja eglanteria* (Luer et al., 2007), *Leucoraja ocellata* (Maxwell et al., 2008), *Urobatis halleri* (Babel, 1966), and *Rhinoptera bonasus* (this study), pectoral fins begin developing as outgrowths from the body wall, as in all vertebrates. In skates and *U. halleri*, the anterior pectoral fins adopt a hook-like morphology and continue developing by growing away from the body (anterodistally) as they fuse to the pharyngeal basket in a rostral progression. The process terminates when the anterior pectoral fins fuse to the rostrum anterior to the mouth. Similarly, the anterior pectoral fins of the cownose ray begin developing posterior to the gill arches and assume a hook-like morphology. In the cownose ray, these hooks correspond to the developing cephalic lobes (**Figure 2A**). As development proceeds, the cephalic lobes unfurl but remain distinguished from the rest of the pectoral fins by a physical "notch," where tissue outgrowth appears to be inhibited (**Figure 2B**). Concurrent with the unfurling of the cephalic lobes, the pectoral fins expand anterodistally and fuse to the body, beginning at the posterior pharyngeal arch and progressing rostrally (**Figure 2C**). Development to this point parallels the process observed in other batoids. However, once the region of the notch fuses at the shoulder near pharyngeal arch I, two processes commence in cownose ray pectoral fins that may be unique to myliobatids.

First, pockets of craniofacial tissue (CFT) begin developing lateral to the mouth (**Figure 2D**). These CFT pockets have ectodermal components that extend posteriorly to cover the gill arches, and distally to form a triangular sheet which eventually fuses to ectoderm of the pectoral fins. Second, the pattern of fusion near the notch begins to deviate ventrally to position the cephalic lobes within the CFT pockets on the ventral side of the body near the mouth. As development continues, the CFT pockets expand (**Figure 2E**), envelop (**Figure 2F**) and fuse to (**Figure 2G**) the cephalic lobes in a medial-distal progression that mirrors the caudal-rostral progression of pectoral fin fusion. By stage seven, the embryonic cownose ray has assumed the shape of an adult: fusion of pectoral fins, cephalic lobes, and CFT envelopment is complete, and only allometric growth remains. Surprisingly, the entire process of pectoral fin fusion spans just

FIGURE 2 | Stages of pectoral fin development in the cownose ray, *Rhinoptera bonasus*. Black bars are 5 mm. All pictures are of the ventral side unless indicated. Top of frame is anterior. Inset images do not necessarily come from the same whole specimen shown and may represent different individuals at the same developmental

(Continued)

2–3 weeks during the first month of the eleven-month cownose ray gestation period (J. Swenson, pers. obs.), indicating that the myliobatid body plan is established very early in development.

Cephalic Lobes Develop as Modified Anterior Pectoral Fins

In cownose rays, cephalic lobes do not develop from independent fin buds emanating from the head; rather, they develop as modifications to the anterior pectoral fins, closely resembling the hooks at the anterior of developing skate and ray pectoral fins (Babel, 1966; Luer et al., 2007; Maxwell et al., 2008). However, unlike the anterior pectoral fins of other batoids, cephalic lobes are distinguished from the rest of the pectoral fin by a small region of reduced tissue outgrowth we call the "notch." Although most of the transient morphologies and processes observed during cephalic lobe development are also found in developing pectoral fins of other batoids (e.g., the anterior hook, fusion to the pharyngeal arches and rostrum), the notch has only been observed in cownose rays in association with cephalic lobe development, suggesting that the notch is a defining feature of cephalic lobe specification.

Another distinction between developing pectoral fins in other batoids relative to cownose rays is manifest in the pattern of tissue fusion. Whereas the anterior pectoral fins in other batoids fuse to the rostrum in the same plane as the pectoral fins, in cownose rays, the pattern of fusion involves a distinct ventral deviation while the pectoral fins are fusing to the shoulder region corresponding to the notch. Ultimately, the unfused cephalic lobes are positioned on the ventral side of the body lateral to the mouth and enveloped within pockets of craniofacial tissue. This process may be unique to myliobatids with ventrally-positioned cephalic lobes.

Shared Gene Expression Patterns in Batoid Paired Fin Development and Homology of Cephalic Lobes
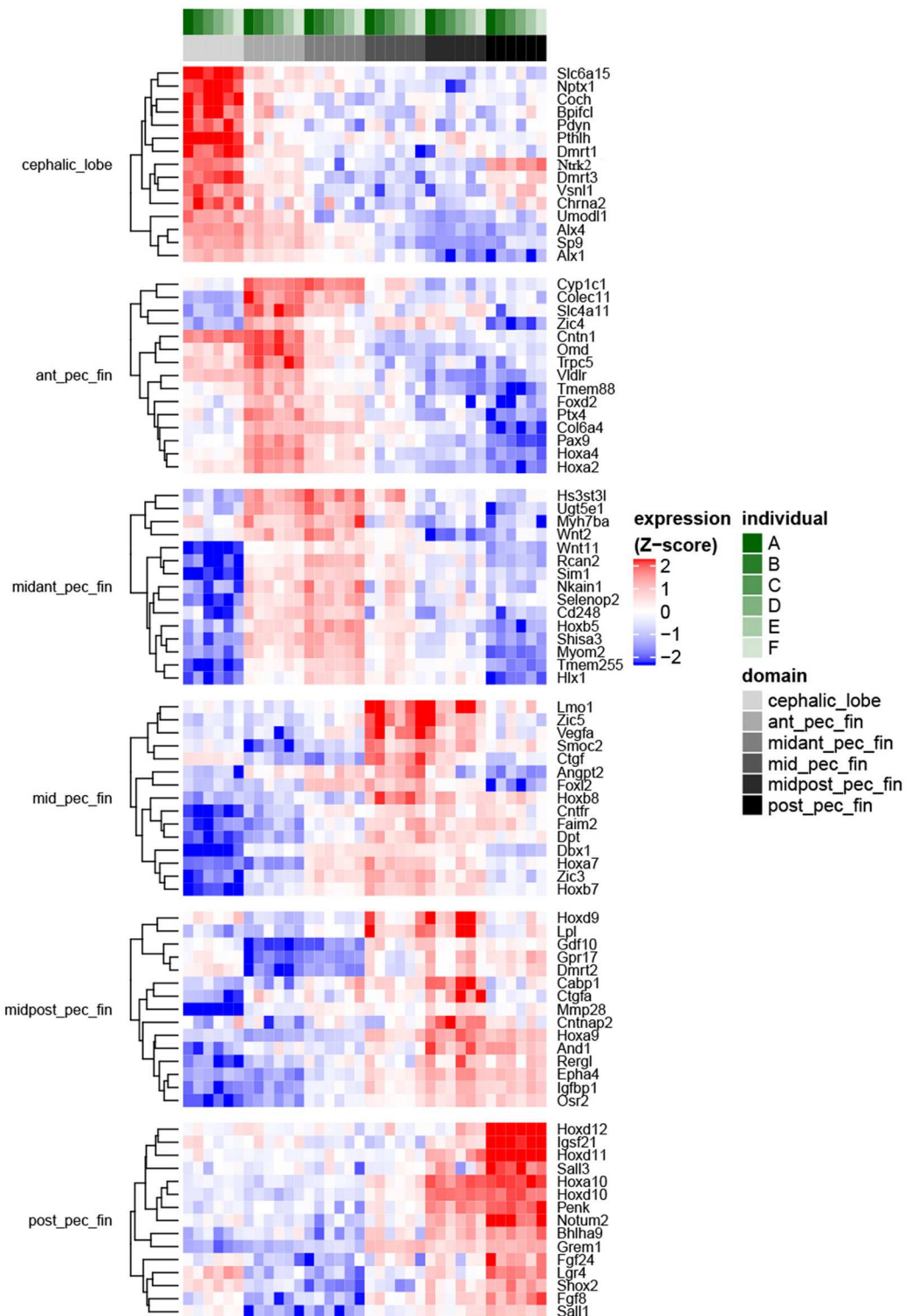
After observing that myliobatid cephalic lobes develop as modified anterior pectoral fins, we used RNA-Sequencing (Wang et al., 2009) to compare gene expression profiles among pectoral fin domains of two batoids—a rajid skate (*Leucoraja erinacea*, little skate) and a myliobatid ray (*Rhinoptera bonasus*, cownose ray)—to determine whether homologous genetic underpinnings pattern the pectoral fins of these species and to reveal patterns that may be shared among divergent batoid lineages.

Using *de novo* transcriptome assembly and reciprocal-BLAST annotation with multiple vertebrates including *Danio rerio*, we confirmed expected gene expression patterns associated with the specific tissue domains dissected in *R. bonasus* (**Figure S2**). We then evaluated differential expression of genes in each *R. bonasus* pectoral fin domain relative to the others (**Figure 3**; **Table S4**). We found expression of *Hoxd10-12* and *Grem1* to be confined to the posterior pectoral fin, while *Hoxd9* and *Hoxa9* were most highly expressed in the mid-posterior pectoral fin, consistent with canonical patterning of paired appendages in vertebrates (Khokha et al., 2003; Zákány et al., 2004; Freitas et al., 2007; Ahn and Ho, 2008; **Figure 3**). We found genes associated with posterior patterning of pectoral fins in *L. erinacea* enriched in the posterior of *R. bonasus* pectoral fins as well (**Figure 4**, green dots), confirming that our samples were undergoing similar patterning processes and that the stages were comparable with respect to fin development (though note that the posteriorly expressed *Ler-Grem1* transcript did not assemble well and was therefore not detected). Further, we confirmed that expression of *Tbx4* and *Tbx5*, genes which define developing pelvic and pectoral fins in vertebrates, respectively (Gibson-Brown et al., 1998; Ahn et al., 2002), exhibited expected expression profiles in *R. bonasus* (for example, average counts per million in *Tbx4* was >20x higher in the pelvic fin (280) when compared to pectoral fin domains (10); whereas the opposite was true with *Tbx5*). Taken together, these observations indicate that the cownose ray RNA-Seq data reliably show domain-specificity and indicate that similar processes drive development of the posterior pectoral fins of skates and myliobatids.

To evaluate homology between the anterior pectoral fin regions of myliobatid stingrays and rajid skates based on shared expression of anterior patterning genes, we identified genes known to be associated with patterning anterior regions of paired appendages and examined their expression in the anterior pectoral fin region of *L. erinacea* (anterior third, hereafter

FIGURE 3 | Fin domain-specific expression patterns highlight both ancestral and derived developmental programs in *R. bonasus*. Differential expression was assessed via the quasi-likelihood f-test (refer to Methods). The heatmap represents genes with transcripts most strongly up-regulated in each of six sampled fin domains when contrasted with the other domains (FDR < 0.001, top 15 genes by log2FC are shown). Transcripts that were up-regulated in more than one domain were assigned to the single domain with the highest expression. Also, transcripts assigned to different isoforms of *D. rerio* genes (i.e., "a" vs. "b") were summarized such that only those with the largest expression differences between domains are shown.

FIGURE 4 | Homologous patterning mechanisms of the anterior and posterior fins of cownose ray and little skate embryos are evident in transcriptomic comparisons. Volcano plots depicting differentially expressed genes in stage 3 *R. bonasus* embryos and stage 31 *L. erinacea* embryos. In *R. bonasus*, both the cephalic lobe and anterior domains (Anterior pectoral fin (RboC)) were contrasted with the posterior and mid-posterior domains (Posterior pectoral fin (RboC); see **Figure 1**), in order to make comparisons with the anterior and posterior *L. erinacea* fin domains examined by Nakamura et al. (2015). The left side of each graph represents transcripts biased in the anterior pectoral fin while the right side represents transcripts biased in the posterior pectoral fin. Labeled transcripts include those that are differentially expressed in the anterior pectoral fins of both species (orange), those that are differentially expressed in the posterior pectoral fins of both species (green), and those that have distinct expression patterns in each species (purple). *Six* replicates for each fin domain were used in *R. bonasus* comparisons and two anterior vs. three posterior fin replicates were used in *L. erinacea* comparisons. Transcripts without high quality annotations were removed for clarity, such that only one transcript is reported for each gene.

APF$_{Ler}$) and the comparable region in *R. bonasus* (hereafter APF$_{RboC}$; see **Figure 1**), which includes the cephalic lobes. By comparing ratios of gene expression in these domains relative to the posterior domains (within each species separately), we identified genes that are similarly enriched in the anterior and posterior pectoral fins of *R. bonasus* and *L. erinacea* (**Figure 4**). These include genes that are known to be associated with anterior patterning in fins and limbs such as *Pax9, Alx4, Alx1*, and *Tbx2* (Onimaru et al., 2015) as well as *Foxc2, Runx1, Meis2* and *Dmrt3* (**Figure 4**, orange dots). We also found upregulation of *Hoxa2* and *Hoxd4* in the anterior pectoral fin regions of both species (**Figure 4**), which are likely associated with a batoid-specific AER in this region (Nakamura et al., 2015). Taken together, these data support homology of the anterior pectoral fin regions—which includes cephalic lobes in the Myliobatidae—in two disparate batoid species with distinct morphologies.

By sequencing six pectoral fin domains in the cownose ray instead of three, we added a layer of spatial resolution to our analyses which also revealed fine-scale expression patterns that were not apparent when considering broad domains for comparison with the little skate. This experimental design revealed high levels of expression of *Hoxd8, Ntrk2*, and *Enpp2* at the anterior of developing cownose ray fins corresponding to

the cephalic lobe (**Figure 5**; **Table S5**). In addition, the increased degree of spatial resolution allowed us to find enrichment of *Wnt3* and *Hoxa13* in the anterior region of *R. bonasus* pectoral fins (**Figure 5**), genes which are also enriched in the anterior of *L. erinacea* pectoral fins as indicated by *in-situ* hybridization (Nakamura et al., 2015; Barry and Crow, 2017). *Wnt3* is associated with a novel apical ectodermal ridge (AER) driving outgrowth at the anterior of the *L. erinacea* pectoral fin (Nakamura et al., 2015). *Hoxa13*, a gene involved in patterning novel paired fin domains in batoids (O'Shaughnessy et al., 2015; Barry and Crow, 2017), has overlapping expression with *Wnt3*, suggesting a role for specification of the anterior AER in batoids (Barry and Crow, 2017). Enrichment of *Hoxa13* and *Wnt3* in the cephalic lobes of *R. bonasus* suggests that the anterior AER found in skates is correlated with development of the anterior pectoral fin region (including cephalic lobes) in derived batoids as well. We note that the high expression of these genes in the anterior pectoral fin region of *R. bonasus* was initially masked (see, for example, *Hoxa13* in **Figure 5**), because they are upregulated in the cephalic lobe (red in **Figure 5**) but downregulated in the anterior (blue in **Figure 5**), resulting in signal cancellation when the expression scores for these domains were merged. Importantly, we found no evidence for independent posterior

FIGURE 5 | Fine-scale expression patterns are evident during *R. bonasus* fin development. A representative stage three *R. bonasus* embryo is shown in ventral view with anterior up. The heatmap represents the average read counts for 6 biological replicates of each fin domain (log2-transformed counts per million, CPM), visualized using a column-based z-score standardization. Gene annotations were originally based on annotation to *D. rerio* and were confirmed by hand (see **Table S3** and **Figure S1**).

patterning by 5′ *HoxD* or *Shh* genes in cephalic lobes, supporting the notion that cephalic lobes do not represent a third set of independent paired appendages, but are instead modified anterior pectoral fins.

Modified Gene Expression Patterns Associated With Unique Myliobatid Features

Unique morphological features arise in association with changes to developmental processes and gene expression patterns. Therefore, genes that exhibit distinct expression profiles in *R. bonasus* and *L. erinacea* during development of the pectoral fins may be associated with morphological differences. We have observed that cephalic lobe development is correlated with at least two surprisingly subtle modifications to the process observed in skate pectoral fins: establishment of a "notch" that defines the cephalic lobe-pectoral fin boundary, and a dorsoventral deviation during pectoral fin fusion. Because pectoral fin fusion is common to both skates and cownose rays, it is difficult to identify genes associated with fusion disparities using our comparative approach. However, we were able to identify candidate notch genes by searching our DGE results for genes that have different expression patterns in the APF$_{RboC}$

and the APF$_{Ler}$ relative to the posterior pectoral fin (i.e., up or downregulated in the anterior of one species but not the other, see pink genes in **Figure 4**). We then searched the literature for functional data related to these genes in other taxa and highlighted those known to be associated with processes that likely underlie development of the notch (e.g., cell proliferation, AER signaling) or the *compagibus laminam* (e.g., calcification, chondrogenic fusion) in **Table 3**.

Using these criteria, we identified six genes that may be associated with distinct morphologies at the anterior region of the pectoral fins of *R. bonasus* and *L. erinacea* (**Table 3**). *Dkk1* is a known inhibitor of the *Wnt* signaling pathway (Glinka et al., 1998; Fedi et al., 1999; Niida et al., 2004), while *Vsnl1* downregulates β-catenin—an important component of canonical *Wnt* signaling—in mouse kidneys (Ola et al., 2011). These genes are upregulated in *R. bonasus* but not *L. erinacea* and may have a role in notch development by inhibiting AER-signaling in this region. Alternatively, *Lhx2* and *Msx1* promote AER signaling and appendage outgrowth during development (Pizette et al., 2001; Lallemand, 2005; Tzchori et al., 2009). These genes are enriched at the anterior of *L. erinacea* and not *R. bonasus*; downregulation of these genes in *R. bonasus* may preclude AER-driven outgrowth at the notch. *Omd* is associated with biomineralization (Kawada et al., 2006; Tashima

TABLE 3 | Genes that are putatively associated with development of pectoral fin modifications in Myliobatids.

Gene	LogFC	Adjusted *p*-value	LogFC	Adjusted *p*-value	Known roles	References
	R. bonasus		*L. erinacea*		Putatively associated with development of the "notch"	
Dkk1	2.06	7.4E-07	Not DE	Not DE	Inhibits Wnt signaling	Glinka et al., 1998; Niida et al., 2004
Vsnl1	2.39	1.6E-06	Not DE	Not DE	Associated with β-catenin signaling in mouse kidneys	Ola et al., 2011
Msx1	Not DE	Not DE	1.12	3.0E-03	Downstream of BMP during AER induction; Associated with proper AER maturation	Pizette et al., 2001; Lallemand, 2005
Lhx2	Not DE	Not DE	1.27	2.8E-03	Regulates *Fgf10* signaling in mesenchyme to maintain proximo-distal outgrowth	Tzchori et al., 2009
	R. bonasus		*L. erinacea*		Putatively associated with development of the *compagibus laminam*	
Omd	1.78	9.3E-14	Not DE	Not DE	Contributes to biomineralization; Regulates diameter and shape of collagen fibrils	Kawada et al., 2006; Fonseca et al., 2014; Tashima et al., 2015
Bco2	Not DE	Not DE	4.5	1.9E-05	Catalyzes endogenous retinoic acid synthesis	von Lintig et al., 2005

*Candidate genes were identified based on different expression patterns in the anterior pectoral fins of little skate and cownose ray relative to the posterior, indicating a potential association with taxon-specific traits. Comparisons were based on the anterior pectoral fin (APF) vs. the posterior pectoral fin (PPF) (see **Figure 1**). Genes were included in this table if they were represented in both transcriptomes, differentially expressed in the APF of one species and not the other, and known to be involved with processes putatively associated with development of the notch (e.g., cell proliferation) or compagibus laminam (e.g., increased fin thickness).*

et al., 2015) and may contribute to development of the thicker fin rays in the *compagibus laminam* of myliobatids. RNA-Sequencing is a powerful tool for identifying genes that may be associated with novel or modified features during development; however, functional studies would be necessary to validate these associations.

Are Paired Fin Modifications Such as Cephalic Lobes and Claspers Specified by Similar Gene Expression Patterns?

While the CFT pockets and cephalic lobes are fusing at the anterior of the pectoral fins in cownose rays, specification of claspers begins at the posterior pelvic fins where they are distinguished by a notch that is reminiscent of the notch that occurs between the cephalic lobes and pectoral fins (see **Figures 2B,D**). As the claspers differentiate from the pelvic fins, they assume a paddle shaped morphology (**Figures 2D,E**) before curling into tubes (**Figures 2F,G**), transiently resembling the curled resting state of cephalic lobes in *Mobula*. As both claspers and cephalic lobes are modified fin domains supported by fin rays, the morphological similarities we observed during development led us to ask whether similar molecular processes could underlie development of these features.

In addition to the pectoral fin domains described above, we sequenced the transcriptome of pelvic fins of the six stage three cownose rays. At this stage, clasper morphogenesis has not yet begun, but we were able to diagnose sex based on expression of genes known to be exclusively expressed during clasper development in males (i.e. not expressed in pelvic fin development of females) such as *Hoxd12*, *Hoxd13*, *Hand2* (O'Shaughnessy et al., 2015) and *Hoxa13* (Barry and Crow, 2017). We confirmed statistically significant enrichment of *Hoxd13*, *Hoxa13*, and *Hand2* in the pelvic fins of three of our samples

TABLE 4 | Genes differentially expressed in the pelvic fins of *R. bonasus* males (*n* = 3) relative to females (*n* = 3) prior to clasper morphogenesis (adj. *p*-values < 0.05).

Gene	logFC	Adjusted *p*-value
Hoxd13	3.85	0.010
Sall1	3.11	0.009
Cdh23	3.02	0.029
Hoxa13	2.89	0.000
Sall3	2.46	0.002
Kit	2.01	0.029
Hand2	1.82	0.036
Six2	1.54	0.000
Cyp7a1	1.49	0.001

relative to the others (**Table 4**), indicating that we had sampled three males and three females. We attributed other differentially expressed genes between male and female pelvic fin domains to clasper development, finding enrichment of *Sall1*, *Cdh23*, *Sall3*, *Kit*, *Six2*, and *Cyp7a1* in male pelvic fins, adding to the repository of genes likely to be associated with clasper development (but note that *Sall1* is expressed in posterior pectoral fins as well, see **Figures 3, 5**).

Interestingly, we found enrichment of five genes in both claspers and cephalic lobes, suggesting a redeployment of outgrowth and patterning pathways during development of these paired fin modifications. In addition to their enrichment in male pelvic fins relative to female, *Hand2* and *Sall1* are enriched in the cephalic lobes relative to the adjacent pectoral fin domains (**Table S5**). *Ntrk2* is similarly upregulated in claspers (logFC = 1.54, *p*-value = 0.0002, adj. *p*-value = 0.13) and highly expressed in cephalic lobes (**Table S4**). Finally, *Hoxa13* is expressed specifically in developing claspers of male pelvic fins,

and is also expressed in anterior pectoral fins of the little skate (Barry and Crow, 2017) and cownose ray (**Figure 5**). Each of these patterns was obscured when examining broad domains in the cownose ray due to low expression in the domain adjacent to cephalic lobe. However, our experimental design allowed us to detect enrichment of these genes specifically in the anterior pectoral fin region corresponding to cephalic lobes (**Figure 5**). Although we did not detect enrichment of putative notch genes (those outlined in **Table 3**) in the pelvic fins of stage three males, this may simply reflect temporal differences in notch development, as the cephalic lobe notch is clearly defined at stage three, while the pelvic fin notch does not appear until stage four (**Figure 2D**). Intriguingly, androgen receptor (*AR*), another gene associated with clasper specification (O'Shaughnessy et al., 2015), was also upregulated in the cephalic lobe relative to every other pectoral fin domain ($p < 0.01$) (see **Figure 5** and **Table S5**). *AR* was not represented in the *L. erinacea* transcriptome, so the possibility remains that *AR* expression at the anterior pectoral fin may be common among batoids. Future studies examining expression of genes associated with clasper development in the pectoral fins and cephalic lobes/APF of batoids may reveal that these paired fin modifications rely on similar developmental-genetic pathways for specification of novel domains.

DISCUSSION

Manta rays and their relatives (Myliobatidae) have functionally partitioned feeding and swimming behaviors into distinct pectoral fin regions which have been modified to optimize these specific tasks. Here, we have examined the morphological and molecular mechanisms underlying development of these modified fin domains in a representative myliobatid, the cownose ray (*Rhinoptera bonasus*), with a focus on the evolution and development of cephalic lobes - a derived functional trait unique to myliobatids. We have identified developmental processes that are conserved among batoids as well as novel processes and morphologies that are unique to myliobatids with cephalic lobes. We provide evidence supporting homology of myliobatid cephalic lobes with the anterior pectoral fins of rajid skates and suggest candidate genes that may be associated with development of the notch and the *compagibus laminam*, two features that are unique to the anterior pectoral fins of myliobatids.

Myliobatid Cephalic Lobes Are Homologous to the Anterior Pectoral Fins of Skates

Batoids are distinguished from other cartilaginous fishes by their disc-shaped pectoral fins, which are dorsoventrally flattened and extend anteriorly to fuse to the rostrum (Last et al., 2016). Species of the family Myliobatidae exhibit pectoral fin modifications during development that result in cephalic lobes. Until this study, the process by which cephalic lobes develop, including their physical attachment to developing pectoral fins, had not been documented. Interestingly, the presence of cephalic lobes is not correlated with an increase in the number of fin rays articulating to the propterygium in myliobatids (Hall et al., 2018), as would

be expected if cephalic lobes were novel additions to ancestral anterior pectoral fins. Rather, the morphological observations and developmental gene expression patterns presented here suggest that cephalic lobes of myliobatids are homologous to the anterior pectoral fin of the little skate–a representative batoid lacking cephalic lobes. These data indicate that the mechanisms underlying anterior expansion of the pectoral fins were present in the common ancestor of skates and rays. The primary difference between developing cownose ray cephalic lobes and skate pectoral fins is the presence of a notch that defines cephalic lobes by separating the anterior pectoral fin into distinct domains. This subtle developmental modification is associated with specification of a distinct region - the cephalic lobe - that is optimized for feeding. The evolution of cephalic lobes is likely associated with modifications to the rest of the pectoral fins that permitted the evolution of oscillatory swimming (a.k.a. underwater flight) and adaptation to a pelagic or bentho-pelagic lifestyle in the Myliobatidae.

Molecular Development of Derived Myliobatid Features

While cephalic lobes are homologous with anterior pectoral fins in skates, there are differences in developmental gene expression patterns associated with unique morphological modifications in myliobatid pectoral fins. Comparative transcriptomics using RNA-sequencing is limited in its capacity for identifying genes that are correlated with distinct phenotypes, as it does not include functional information. Nevertheless, by searching the data for genes that are up or down-regulated at the anterior of one species and not the other, we identified candidate genes that may be associated with myliobatid-specific features such as the *compagibus laminam* and development of the notch (**Table 3**; **Table S6**).

The expression pattern of osteomodulin (*omd*) is particularly intriguing. In *R. bonasus*, expression of *omd* is concentrated at the anterior of the pectoral fin, posterior to the cephalic lobe in the region of the *compagibus laminam* (**Figures 3**, **5**). The pattern differs from *L. erinacea*, where *omd* is slightly upregulated at the posterior (**Figure 4**). Due to its known role in biomineralization and enriched expression in a pectoral fin domain of *R. bonasus* that exhibits increased calcification, we hypothesize that *omd* may be associated with development of the *compagibus laminam*, a plate of nearly fused and highly calcified fin rays at the anterior of myliobatid pectoral fins (Schaefer and Summers, 2005; Hall et al., 2018) which likely contributes to lift generation during oscillatory swimming.

The notch, a developmental feature that maintains a clear delineation between the cephalic lobe and the rest of the pectoral fin, likely develops as a region that is marked for reduced tissue outgrowth, or interruption of the AER, relative to the ancestral state. Once we identified genes with different expression patterns in the anterior pectoral fins of *R. bonasus* and *L. erinacea*, we queried this list for genes that have been shown to be associated with developmental pathways and processes that may be pertinent to notch formation (e.g., *Wnt* signaling, cell proliferation, apoptosis). Of the identified candidate genes,

Dkk1, *Msx2*, and *Lhx2* are compelling candidates for notch induction. *Dkk1* is a known *Wnt* inhibitor. *Wnt* signaling is an important component of AER induction and maintenance (ten Berge et al., 2008). Upregulation of *Wnt* inhibiting genes at the notch could provide a mechanism by which AER-facilitated outgrowth could be stunted in the notch while continuing in the adjacent regions. Alternatively, downregulation of genes such as *Msx1* and *Lhx2* that promote AER maintenance could have a similar effect, resulting in reduced cell proliferation (**Table 3**). Though we did not identify any candidate notch genes with roles in apoptosis, apoptosis could also be a mechanism by which a notch could form in developing fins. If apoptotic pathways were activated in the notch, AER signaling could occur as a continuous strip throughout the anterior pectoral fin (including cephalic lobe), while an increase in cell death at the notch could prevent tissue outgrowth from keeping pace with adjacent areas. Further research into notch development is needed to confirm the underlying mechanisms, perhaps via functional studies that attempt to induce a notch in a more tractable batoid species such as the little skate (*Leucoraja erinacea*) via ectopic expression or deletion of the genes highlighted in **Table 3**.

Development of the anterior region of myliobatid pectoral fins appears to involve a unique combination of Hox gene expression and redeployment of posterior modules. Though most *HoxD* genes exhibit canonical expression patterns in the pectoral fin of *R. bonasus* (**Figure 5**), expression of *Hoxd8* is conspicuously enriched in the APF/cephalic lobe of *R. bonasus* but not *L. erinacea* (**Figures 4**, **5**), suggesting that there may be a myliobatid-specific Hox code at the anterior of the pectoral fin. In addition, *Enpp2* (aka *autotaxin*), is a known target of *Hoxa13* (Williams et al., 2005) and may contribute to AER induction in the anterior pectoral fins of batoids (this gene was not represented in the *L. erinacea* transcriptome), or specification of cephalic lobes in myliobatids. Mirroring the pattern of *Enpp2*, *Ntrk2* expression is enriched in both the cephalic lobes and the posterior pectoral fins of *R. bonasus*, as are *Wnt3* and *Hand2* (**Figure 5**). By revealing genes with expression patterns that are mirrored at the anterior and posterior of *R. bonasus* pectoral fins, our domain-specific data suggest that these regions share a handful of common patterning mechanisms, which may contribute to development of boundaries in paired fins.

Developmental Variations in Batoids

Modifications to outgrowth and fusion processes may contribute to the morphological variation observed at the anterior pectoral fins among myliobatid taxa. For example, the single fused cephalic lobe of mature bat rays is found in the same plane as the pectoral fin, indicating that the observed deviation during pectoral fin fusion in the region of the notch in cownose rays is lacking in bat rays (**Figures 6A,B**). This appears to be due to a dorsoventral shift in the pattern of fusion at the notch region in cownose rays and other myliobatid species. While species in the myliobatid genera *Rhinoptera*, *Mobula*, and *Aetobatus* lack skeletal elements in the shoulder (corresponding to the notch), some bat rays of the genus *Myliobatis* display a radiation of stunted fin rays in this region between the cephalic lobe and pectoral fin (**Figures 6C,D**). We hypothesize that, relative to
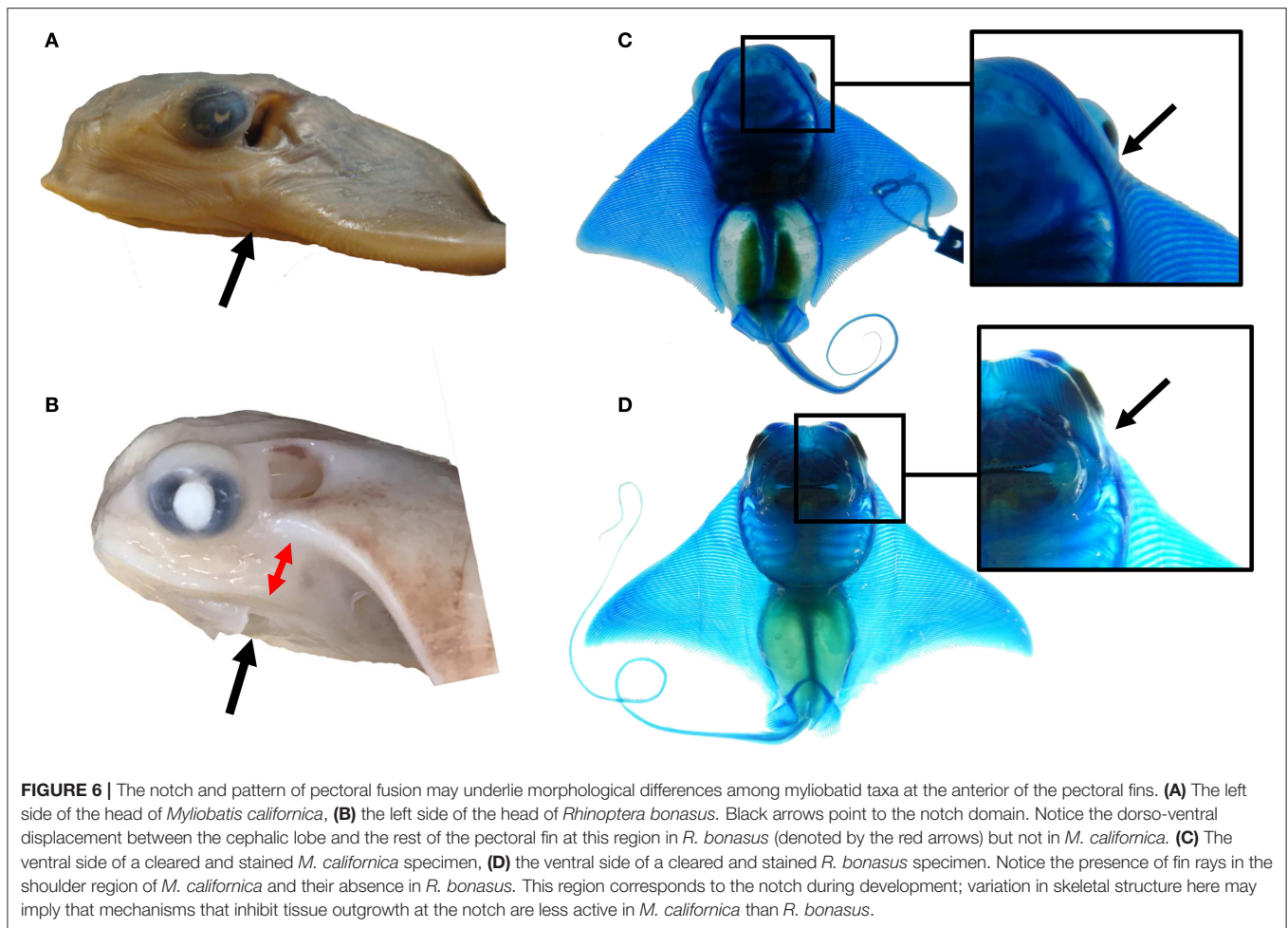
other myliobatids, these bat rays exhibit weak or temporally constricted expression of the genes responsible for suppressing tissue outgrowth in the notch. Combined, these observations suggest that the molecular processes driving notch formation and the pattern of fusion may vary among myliobatid taxa, with representation of intermediate phenotypes.

Aberrant phenotypes reflecting incomplete pectoral fin fusion have been observed in various batoid clades, including the Myliobatidae (Ramírez-Amaro et al., 2013), Rajidae (Prado et al., 2008), Torpedinidae (Mnasri et al., 2010), Dasyatidae (Prado et al., 2008; Ramírez-Hernandez et al., 2011), Urotrygonidae (Mejía-Falla et al., 2011), Potamotrygonidae (Oldfield, 2005a,b), Rhinobatidae (Bornatowski and Abilhoa, 2009), and Gymnuridae (Narváez and Osaer, 2016). This prevalent deformity was first described over a century ago (Gill, 1896) and has since been termed the "batman" morphology (Oldfield 2005a; Oldfield 2005b). After observing that a two-step process consisting of distinct phases of outgrowth and fusion underlies development of the anterior pectoral fins in cownose rays, we suggest that the batman morphology may occur when the molecular mechanisms driving outgrowth proceed normally, while the mechanisms underlying fusion are interrupted. It has been suggested that the batman morphology may be associated with exposure to pollutants (Heupel et al., 1999), which could disrupt developmental-genetic processes (Tomanek, 2011; Varotto et al., 2013).

Are Paired Fin Modifications Such as Cephalic Lobes and Claspers Specified by Similar Gene Expression Patterns?

Claspers are male reproductive organs in cartilaginous fishes, which are modifications to the posterior pelvic fins in extant taxa. Morphologically, clasper formation is preceded by development of a notch between the budding claspers and pelvic fins, reminiscent of the notch that develops between the cephalic lobes and the rest of the pectoral fins. This led us to wonder if specification of cephalic lobes in anterior pectoral fins represents a mirror image of clasper specification in posterior pelvic fins, and if we could find evidence for co-option of this ancient developmental pathway in other modified fin domains.

Transcriptomic data revealed that genes known to be involved in clasper specification are indeed enriched in developing cephalic lobes. Barry and Crow (2017) found that *Hoxa13* is expressed exclusively in the claspers of the little skate, and not expressed in female pelvic fins. Variation in the pattern of *Hoxa13* expression is often associated with the evolution of novel features (Crow et al., 2009; Archambeault et al., 2014; Barry and Crow, 2017), including the autopod (Sordino et al., 1995; Yokouchi et al., 1995; Fromental-Ramain et al., 1996; Metscher et al., 2005), making it a likely candidate for specification of derived batoid features as well. During clasper development, *Hand2* is regulated by hormonal input via upregulation of *AR* (O'Shaughnessy et al., 2015); it was suggested that the evolution of claspers was facilitated by hormonal input to fin developmental networks following the evolution of androgen response elements at the *Hand2* locus (O'Shaughnessy et al., 2015). Interestingly,

FIGURE 6 | The notch and pattern of pectoral fusion may underlie morphological differences among myliobatid taxa at the anterior of the pectoral fins. **(A)** The left side of the head of *Myliobatis californica*, **(B)** the left side of the head of *Rhinoptera bonasus.* Black arrows point to the notch domain. Notice the dorso-ventral displacement between the cephalic lobe and the rest of the pectoral fin at this region in *R. bonasus* (denoted by the red arrows) but not in *M. californica.* **(C)** The ventral side of a cleared and stained *M. californica* specimen, **(D)** the ventral side of a cleared and stained *R. bonasus* specimen. Notice the presence of fin rays in the shoulder region of *M. californica* and their absence in *R. bonasus.* This region corresponds to the notch during development; variation in skeletal structure here may imply that mechanisms that inhibit tissue outgrowth at the notch are less active in *M. californica* than *R. bonasus.*

both *Hand2* and *AR* are similarly enriched in developing cephalic lobes. In conjunction with high levels of *Ntrk2* and *Sall1* expression in both claspers and cephalic lobes (relative to the adjacent fin domain; see **Figure 5**, **Table S5**), our data support an intriguing hypothesis: namely, that cephalic lobes, which are anterior pectoral fin modifications, may have evolved by co-opting developmental pathways associated with the evolution of claspers - posterior pelvic fin modifications in male cartilaginous fishes.

CONCLUSIONS

We have used interspecies comparative transcriptomics to shed light on the evolution and development of paired fins in a batoid family with derived features (Myliobatidae). We found that myliobatid pectoral fins develop via a two-step process in which they first extend anterodistally and subsequently fuse lateral to the gill arches and rostrum, while cephalic lobes develop as modified anterior pectoral fins that fuse anterior to the mouth. Morphologically, the feature that distinguishes the myliobatid pectoral fin from that of other batoids during early fin development is the notch, while ventral deviation during fusion

of the pectoral fins may be unique to derived myliobatid taxa with ventrally-positioned cephalic lobes. We find no evidence supporting cephalic lobes as independent paired appendages; rather, cephalic lobes are modified anterior pectoral fin domains that share homology with the anterior pectoral fins of skates while also exhibiting distinct expression patterns of a handful of candidate genes, including some that are known to be associated with clasper development. This research represents a foray into the frontiers of evolutionary developmental biology: by applying advanced molecular techniques to elucidate the evolution and development of a non-model organism with derived functional traits, we have learned that the distinctive myliobatid body plan evolved via subtle changes to ancestral developmental pathways.

DATA AVAILABILITY

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the San Francisco State University Institutional Animal Care and Use Committee (IACUC) under protocol A15-09R2. The protocol was approved by the IACUC.

AUTHOR CONTRIBUTIONS

JS and KC designed research and wrote manuscript. JS and RF collected samples and field data. JS performed labwork. JK assembled transcriptome and performed DGE analyses. All authors edited and approved final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00181/full#supplementary-material

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