Ontogeny of myosin isoform expression and prehensile function in the tail of the grey short-tailed opossum (*Monodelphis domestica*)

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ABSTRACT

Terrestrial opossums use their semi-prehensile tail for grasping nesting materials as opposed to locomotor maneuvering. The objective of this study is to relate the development of this adaptive behavior with ontogenetic changes in myosin heavy chain (MHC) gene regulation and isoform expression in the tail from 3 weeks to adulthood. *Monodelphis domestica* is expected to demonstrate a progressive ability to flex the tail up to age 7 months, when it will have full use of its tail and should exhibit routine nest construction. We hypothesize that juvenile stages (3–7 months) will be characterized by retention of the fast neonatal isoform (MHC-Neo), along with large expression of fast MHC-2X and 2B, which will transition into slow MHC-1 and fast 2A isoform fibers as tail development progresses. This hypothesis was tested using qPCR to quantify and compare gene expression of each isoform to its protein content found by gel electrophoresis and densitometry. These data were paired with video observations of nesting activity in an age-matched sample of each age group studied. Shifts in regulation of MHC gene transcripts matched well with isoform expression. Notably, mRNA for MHC-Neo and 2B decrease, largely resulting in no isoform translation after age 7 months, whereas mRNA for MHC-1 and 2A increase and this corresponds with increasing protein content for these isoforms into late adulthood. A critical growth period for isoform transition is observed between 7 and 13 months, and this correlates with increased use of the tail for nest construction as adults. Ontogenetic shifts from faster- to slower-contracting isoforms may be associated with muscle 'tuning' for the repetitive nest remodeling tasks requiring sustained low force contractions of the caudal flexors.

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DEDICATION

I dedicate this Thesis to my family, especially my mother Toni and my father Danny for their unfaltering support, both emotionally and financially, throughout my academic career and during the completion of this Thesis.

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INTRODUCTION

Monodelphis is the most specious genus of the family Didelphidae (subfamily: Didelphinae; Tribe: Marmosini), comprising 21 recognized species (Voss and Jansa, 2009) of small terrestrial opossums with short tails, diminutive ears, and variable pelage patterns (Gardner et al., 2005; Solari, 2010; Vilela et al., 2010). The gray short-tailed opossum, *Monodelphis domestica* (Wagner, 1842), is considered to be representative of the genus (Mikkelsen et al. 2007; Pavan et al. 2014). It primarily inhabits the neotropical rainforest regions surrounding the Amazon Basin in eastern, southern, and central Brazil (Emmons and Feer, 1990; Eisenberg and Redford, 1999), where it uses thorn scrub and granitic outcrops as common habitats on the forest floor. Notably, *M. domestica* lacks a marsupium, is habitually terrestrial, and retains a semi-prehensile tail (Macrini, 2004), which is nearly half the length as the head and body (total body length: ~15 cm), giving this species an average relative tail length of 0.5 (Rupert et al., 2014). The tail is used to grasp and carry materials (e.g., leaves, grasses, bark, and snake skin) for nest construction (Unger, 1982; Grizimek, 1990), and *M. domestica* remodels its nest several times per day in response to environmental temperature fluctuations (Macrini, 2004). Thus, employment of the semi-prehensile tail to build protective nests is highly instrumental to its thermoregulatory strategy (Nowak, 1999).

Despite the utility of tail prehension for nest building function that is critical to survival in opossums, little is understood about the development of this adaptive behavior or the corresponding ontological changes in caudal muscle physiology. However, studies of postnatal development in the hind limb muscles of mice and rats (e.g., Caplan et al., 1983; Whalen et al., 1984; Schiaffino et al., 1988; DeNardi al et., 1993; Agbulut et al., 2003) provide insight into transitions in myosin heavy chain (MHC) isoform composition that are expected with mechanical loading by the following key observations: 1. a shift from embryonic/neonatal MHC isoforms to the adult fast MHC-2A isoform, and 2. the up-regulation fast MHC genes and expression of adult MHC-2A, 2X, and 2B isoform fibers with weight-bearing activity. The developmental isoforms are formed during the prenatal stages when essentially no load is placed on muscles (Schiaffino and Reggiani, 2011). Therefore, the expression of developmental MHC isoforms throughout ontogeny may provide a reliable physiological signal for timing developmental changes associated

with increasing use of an appendage not involved in weight-bearing or locomotor maneuvering.

The objective of this study is to evaluate the ontogeny of MHC isoform expression in the tail flexor musculature of *M. domestica* from age 22 days to late adulthood. At birth, the tail is not developed and the newborn opossum is able to use only its forelimbs for movement (Macrini, 2004). Weight bearing on all four limbs is estimated to occur during the sixth week of development, followed by a progressive ability to flex the distal tail up to age seven months, when *M. domestica* is expected to have full use of its semi-prehensile tail and exhibit routine nest construction (Pflieger et al., 1996). Previously, we determined myosin fiber types in the tail of adult *M. domestica* and found relatively large distributions of both slow MHC-1β and fast, oxidative MHC-2A/X hybrid fibers, in addition to a modest percentage of pure MHC-2A fibers (Rupert et al., 2014). These data provide important details about the sub-divisions of fast MHC isoforms to more accurately interpret tail function and suppose that slower-contracting caudal muscles may be specialized for the repetitive nest remodeling tasks requiring low force contractions. This study builds on our previous work by relating MHC isoform content with both MHC gene regulation and nest construction behavior to test that hypothesis. Specifically, we predict that juvenile stages of development (3–7 months) will be characterized by retention of the neonatal isoform (MHC-neo), along with large expression of the fast isoforms MHC-2X and 2B, that will transition to MHC-2A and slow MHC-1β, respectively, as tail development and use progresses into adulthood. Quantifying changes in MHC expression and gene regulation as it relates to the development of tail prehensility helps answer questions about genomic fiber type specialization in didelphids, and more generally, improves our understanding of how appendage muscles are developed and physiologically modified with use during critical growth periods.

MATERIALS AND METHODS

Animals

A total of 31 gray short-tailed opossums (*M. domestica*) were used in this study. Specifically, twenty two (*N*=3 neonates, *N*=15 juveniles, and *N*=4 adults) animals were used for genomic and proteomic analyses, and nine (*N*=6 juveniles and *N*=3 adults)

animals were used for behavioral analysis. All animals (male and female) were obtained in collaboration with the Texas Biomedical Research Institute in San Antonio, TX. Animals were euthanized by CO_2 asphyxiation and upon confirmation of death (by cervical vertebral dislocation), fresh muscle tissue was harvested from their tails (see below) and then shipped on dry ice to Youngstown State University (YSU) for analysis. All experimental procedures followed protocols approved by the YSU Animal Care and Use Committee (IACUC: 01-12 and 04-12).

Muscle harvesting and Protein preparation

Caudal muscle tissue was harvested immediately post-mortem. The tails were divided into 'proximal', 'transitional', and 'distal' regions for muscle dissection (Hazimihalis et al., 2013; Rupert et al., 2014). A small tissue block of the *m. flexor caudae longus* (FCL) from each tail region was sampled (stored at -80°C) and prepared for electrophoresis by the following method: flash freezing in liquid nitrogen, grinding to powder, homogenizing 50 mg of muscle powder in 800 µl (ratio 1:16) of Laemmli buffer with 62.5 mM Tris (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, and 2.3% SDS (Laemmli, 1970), and centrifugation of the muscle homogenates at 13k rpm for 10 min. Protein samples for gel loading were diluted to a final protein concentration of ~0.125 µg/µl with gel sample buffer containing 80 mM Tris (pH 6.8), 21.5% glycerol, 50 mM DTT, 2.0% SDS, and 0.1% bromophenol blue (Mizunoya et al., 2008). Samples were then heated (95ºC) for 5 min and then loaded on gels for electrophoresis. Mammalian heart (left ventricle) and rat tibialis anterior (TA) were prepared by the same methods to serve as a band standards for MHC-1β and MHC-2B, respectively, in the gels.

Protein analyses: SDS-PAGE and densitometry

MHC isoforms were separated using established methods (Talmadge and Roy, 1993) performed with slight modifications (Mizunoya et al., 2008; Rupert et al., 2014). Briefly, the acrylamide-*N,N'*–methylenebisacrylamide (Bis) ratio of the gels was 50:1, with the total acrylamide percentage equaling 8% and 4% in the separating gel and stacking gel, respectively. The electrode buffer was 50 mM Tris (pH 8.3), 75 mM glycine, and 0.5% SDS; upper buffer was 6X the concentration of the lower buffer and also contained 0.12% β-mercaptoethanol. Electrophoresis was run on a mini-PROTEAN Tetra system

(Bio-Rad, Hercules, CA USA) at 140 V for 24 h at 4°C (Talmadge and Roy, 1993; Mizunoya et al., 2008). For densitometry analysis, gels were loaded with 1 μg of protein per lane and stained with silver for visualization of the MHC isoforms. Stained gels were imaged using a Pharos FX Plus system (Quantity One software: Bio-Rad). MHC isoform content was then quantified by densitometry in Image J (v.1.43: NIH). In each gel lane (representing one tail region per individual), band intensity values were summed and used to calculate a percentage for each MHC isoform. Percent MHC isoform composition was averaged across 3–5 measurements made from independent gel experiments.

Genomic analyses: rtPCR and qPCR

MHC bands resolved by SDS-PAGE were sectioned from the gels, placed in tubes containing a 5% acetic acid buffer, and sent to The Ohio State University for protein sequencing of the isoforms. Isoform identity was confirmed by matching the protein sequences with published MHC sequences accessed in the NCBI BLAST databank. Sets of forward and reverse primers were then custom designed using the protein sequences (Table 1), and each set was made specific to unique 20 base pair regions (outside the amplicon) of each MHC isoform by Integrated DNA Technologies (IDT, Coralville, IA USA). Next, frozen tissue blocks (25–30 mg) of FCL were homogenized (on ice) in conical tubes containing RLF lysis buffer (QIAGEN, Valencia, CA USA) and repeated for two cycles (15 sec/cycle). Total RNA was extracted from the muscle homogenate products using an RNeasy® Fibrous tissue Mini Kit (QIAGEN), and the concentration of each RNA sample was measured with a NanoDrop 2000c spectrophotometer (ThermoScientific, Wilmington, DE USA). A portion total RNA was then diluted to a concentration of 20 ng/μl and reverse transcriptase polymerase chain reaction (rtPCR) was run with an iCycler Thermal Cycler (Bio-Rad) using one primer pair (forward and reverse) for each MHC isoform studied to verify primer specificity (Rourke et al., 2004). Specifically, rtPCR was performed using a One Step RT-PCR Kit (QIAGEN) to prepare reaction mixes containing 60 ng of template RNA. Reactions proceeded for a total of 40 cycles (~2 h) following conditions optimized by the kit. A yield of 5 μl from each PCR product was combined with 2 μl of EZ-Vision Three DNA dye (Amresco, Solon, OH USA), electrophoresed on 1.5% agarose gels at 70 V for 25 min, and lastly visualized under UV light to detect MHC bands.

Extracted total RNA was additionally diluted to 50 ng/µl to synthesize a series of cDNA libraries using an iScript cDNA Synthesis Kit (Bio-Rad). The cDNA libraries served as templates for qPCR reactions (Toniolo et al., 2007, 2008). A total of 1.0 ng of synthesized cDNA was amplified by qPCR reactions that proceeded under the following conditions: 3 min of polymerase activation at 95°C, and 15 sec at 95°C (denaturation) followed by 30 sec at 55ºC (annealing/extension) for 40 cycles total. Also, to ensure there were no primer dimers, a melt curve analysis was performed for a 2–5 sec/step cycle, raising the temperature from 55ºC to 95ºC in increments of 0.5ºC for each step. All qPCR reactions were performed in 20 µl of a PCR master mix containing: 10 µl of 2× SYBR® Green supermix (Bio-Rad), 2 µl of each primer (3 µM stocks), 2 µl of cDNA template (0.5 ng/µl), and 4 µl DNase-free H_2O.

Behavioral observations

Video data was collected on an age-matched sample (N=9) of *M. domestica* at the Texas Biomedical Research Institute to quantify their nocturnal nesting activity. Over two separate three day periods, 2–3 digital video cameras (frame rate: 60 Hz) recorded juvenile and adult opossums overnight (1800–0900 h) to document tail behaviors associated with the initiation of tail flexion and nest construction. Each camera was placed 0.70 m away from the enclosures, at a height of 1.10 m, and adjusted at an angle 35 deg to film four enclosures simultaneously (Fig. 1A, B). The frequencies of the desired behaviors (Youlatos, 2008) were quantified using a custom routine in MATLAB (MathWorks, Natick, MA USA). Total percentages of time *M. domestica* displayed nest construction behaviors both with (NT) and without (N0) its tail were calculated for the age groups: 3 mo, 5 mo, 7 mo, 13 mo, and 29 mo. Two tail-specific behaviors were additionally evaluated for their frequencies: flexion of the distal tail (DF) and grasping/carrying of nesting materials (GC).

Data analysis and Statistics

The qPCR reactions were repeated 3x for each MHC isoform and age group sample studied with minimal variability between trials. These data were used to calculate cycle number means for each sample (Fig. 2A). Gene expression (total mRNA) was quantified by generating a series of standard curves for each isoform by using known load

concentrations and mean cycle number (Fig. 2A); data were log transformed and fit with linear least-squares (LLS) regressions to determine the calibration equations (Fig. 2B). Calibration curves for each isoform studied showed high reliability with $R^2 \geq 0.97$. Values of mRNA for each sample were quantified, summed, and then grouped by age and tail region to calculate a percentage of the total mRNA for each MHC isoform. Percentage of both mRNA and MHC content were averaged across each tail region and plotted for each age group to demonstrate the cumulative change trends with development. Expression ratios representing the proportional changes in both mRNA and protein products were additionally calculated to demonstrate the relative changes in MHC expression with each progressive age group across ontogeny.

All data are presented as mean (± s.d.) unless otherwise noted. Means for mRNA and MHC isoform content are shown as bar charts. Tail-specific behavioral frequencies (DS and GC) were plotted with MHC content to determine the relationships between the tail use and MHC isoform expression. Pearson product-moment correlation coefficients (r) for these data were analyzed for statistical significance in R (v. 3.1.0: http://www.r-project.org). Lastly, correlation tests between body mass and MHC content percentage were also performed in R. Significance for all correlation tests was accepted at $p<0.05$.

RESULTS

MHC expression: mRNA and isoform proteins

The FCL of neonate and juvenile *M. domestica* showed expression of the adult MHC-1, 2A, 2X, and 2B isoforms, in addition to the presence of the developmental isoform MHC-Neo band (Fig. 3). In accordance with our previous report on adult Monodelphis (Rupert et al., 2014), MHC-2A and MHC-2X bands were predominant and clearly resolved in each individual, while bands for the fast MHC-2B and slow MHC-1 isoforms were comparatively lighter in their resolution (Fig. 3). A thin band for MHC-Neo migrated to a position just below MHC-2X and was resolved in individuals up to age 7 months, but was absent in gels for all but one adult male opossum. The developmental isoform MHC-Emb was not clearly expressed in any individual or tail region.

The cumulative mean distribution of MHC gene expression (mRNA) in the FCL is shown in Figure 4. In all age groups studied, the mRNA for MHC-1β isoform was the

most abundantly expressed, and generally demonstrated a proximal-to-distal decrease in its percent expression along the length of the tail (Table 2). Notably, there was a progressive increase in mRNA of MHC-1β from age 5 months to adulthood, with MHC-1β accounting for 80.2% of total gene expression by 29 months (Fig. 4). MHC-2A showed the opposite pattern by initially having increased mRNA expression from age 22 days to 5 months, followed by a progressive decrease in percentage mRNA into late adulthood. As an example, the FCL had a mean of 28.6±6.15% for the MHC-2A at 5 months compared with a mean of 16.3±3.40% at 13 months (Fig. 4). For the remainder of the MHC isoforms, overall mRNA expression was relatively low and somewhat variable among age groups and tail region (Table 2). With the exception of the developmental isoforms MHC-Emb and MHC-Neo (22 days), and adult MHC-2B (3 months) early in development, mRNA for the faster-contracting isoforms did not exceed 10% of total MHC gene expression in the FCL (Fig. 4).

Figure 5 shows the cumulative mean distribution of MHC isoform content in the FCL. Beyond age 22 days, for which fast MHC-2B accounted for 23.6% of the total isoform content distribution, the fast MHC-2A and MHC-2X isoforms were consistently expressed in the largest percentages in each age group and region of the tail (Table 3). MHC-2A content decreased modestly from age 22 days (~50%) to mean of 40.6±2.5% at 7 months and after which, increased more appreciably throughout adulthood to its highest mean value of 62.1±2.5% at 29 months (Fig. 5). While percent MHC-2X isoform content initially increased from 10.6% at age 22 days to nearly 30% at 3 months, no clear pattern of relative increases or decreases in the MHC-2X isoform content could be discerned throughout the remainder of ontogeny. Percentage isoform content for MHC-2X was the highest at 7 months with a mean of 37.4±7.2% and the lowest at 29 months with a mean value of 19.3±4.3% (Fig. 5). Also, 7 months was the only age at which the mean MHC content of MHC-2X was similar to that of MHC-2A. Slow MHC-1β was expressed in relatively low percentages, but its total isoform content steadily increased from 3 months to 29 months. As was found for MHC-2A, the isoform MHC-1β had its highest mean value of 18.6±4.2% at 29 months (Fig. 5). Lastly, expression of MHC-2B and MHC-Neo was somewhat variable among individuals in each age group. Overall, isoform content for MHC-2B decreased, while that of MHC-Neo remained similar across ontogeny up to

13 months. The resulting percentages for these fast isoforms were also low (Table 3), and both of these isoforms were generally not expressed in the FCL of adult *M. domestica* beyond age 7 months (Fig. 5). The one exception was the finding of a variable small percentage of MHC-2B and MHC-Neo across the tail regions of an adult male opossum (Table 3). Proportional (relative) changes for both mRNA expression and MHC isoform content across ontogeny are summarized in Figure 6.

Tail behavior: Correlations with MHC and body size

Total nesting activity increased progressively across ontogeny in *M. domestica* (Table 4). Opossums age 13 months nested for nearly 13% of their nocturnal activity. This was highest percentage of nesting activity found in any age group, followed by a total of 5.3% for the 29 month old individual, while the 3 month age sample demonstrated 0% nesting activity (Table 4). Size-scaled representations of opossums in each age group studied along with their relative nesting activity with the tail (NT%) are shown in Figure 1C. Again, opossums age 13 months had the highest NT% (Table 4). The 29 month old individual nested for nearly equal percentages of time with and without the tail (N0%). NO% percentage was higher than that of NT% in both the 5 month and 7 month age samples. Notably, opossums age 7 months nested entirely without their tail (Table 4).

With the exception of the 7 month age sample, there was also a progressive decrease in the frequency of distal tail flexion (DF%) accompanied by an increase in the frequency of grasping/carrying of nesting material (GC%) (Table 5). Opossums age 3 months were only capable of flexing their distal tail, while the 29 month old individual did not flex its distal tail in the absence of nest construction. In general, DF% was high in the juveniles age 3–7 months with little-to-no observations of grasping/carrying nesting material. Opossums age 13 months had the highest total number of observations for tail-specific behaviors corresponding with their relatively high NT%. Figure 7 shows the relationships between percentage MHC isoform content and GC% for the distal tail. Expression of MHC-1β (Fig. 7A) and MHC-2A (Fig. 7B) both increased with tail use and have strong positive correlations. Conversely, the isoforms MHC-2X, 2B, and Neo all showed negative correlations with GC% (Fig. 7C, D, E). The correlations coefficients (r) for each relationship were significant. It is noted that the inverse of these trends were found when percentage MHC content was plotted with DF% (data not shown). Lastly, the

relationships between MHC isoform content and body mass are shown in Figure 8. The isoforms slow MHC-1β (Fig. 8A) and fast MHC-2B (Fig. 8D) showed strong positive and negative correlations, respectively, with body size. The corresponding r values for MHC-1β ($p=0.03$) and MHC-2B ($p<0.01$) were significant. MHC-2A (Fig. 8B) and the MHC-Neo (Fig. 8E) showed no relationship with body size, while percentage content for MHC-2X was positively correlated with body size, although this relationship was not significant ($p=0.33$).

DISCUSSION

This study employed qPCR, SDS-PAGE, and behavioral quantification to correlate the adaptive behavior of nest construction with mRNA regulation and MHC isoform expression. The MHC protein data provide the clearest evidence to substantiate our hypothesis that the overall isoform composition of the tail of juvenile *M. domestica* is fast-contracting. In particular, expression of adult MHC-2B and the developmental isoform MHC-Neo is consistently retained through age 7 months, when opossums are expected to reach sexual maturation. The MHC composition of the FCL then becomes slower-contracting into adulthood with increased tail use and body size. This assertion is directly supported by two sets of observations: (i) Increases in slow MHC-1β content and decreases in fast MHC-2B content are strongly dependent on body size, and (ii) Expression of both MHC-1β and 2A are positively correlated with prehensile function (i.e. increased grasping/carrying of nesting materials), while the fast isoforms MHC-Neo, 2X, 2B are all negatively correlated with tail use for nest construction. Collectively, these findings are suggestive of fast-to-slow transitions (or shifting) in MHC isoform expression with development, and further substantiate our ontological hypotheses for non-weight bearing appendage.

Several factors have been found to contribute to myosin fiber type transition, including the degree of mechanical loading, neuromuscular activity, hormone levels (namely the thyroid hormones), and aging (Pette and Staron, 1997, 2000). Corresponding with changes in these conditions, gene regulation is altered whereby changes in promoters, mRNA transcription, and post-translational modifications all affect MHC expression. Much of these data have come from studies using mouse and rat muscle

models. For example, during the few first weeks after birth, a progressive disappearance of the developmental isoforms MHC-Emb and MHC-Neo is observed, along with up-regulation of the fast isoform genes and consequent increased expression of the MHC-2A, 2X, and 2B isoforms (DeNardi et al., 1993). The mRNA levels shown in Figure 3 are consistent with these previous findings by indicating down-regulation of the developmental isoforms and up-regulation of all adult fast isoforms from age 22 days to 3 months. Data from small rodents also indicate that MHC-Neo is lost more slowly in fibers that transition into ones that express the adult MHC-2A isoform (Schiaffino et al., 1988). This may offer a plausible explanation for the retention of MHC-Neo into early adulthood due to the consistently high percentage content of MHC-2A in the FCL of *M. domestica* across ontogeny.

During the early stages of development, there is no innervation of the tail musculature. Neuromuscular innervation of the FCL is expected to occur at approximately 2 months of age (Pflieger et al., 1996). Moreover, beyond the inability to excite the musculature to contract, the tail is incapable of bearing any type of mechanical loading. Despite the absence of these factors, there is a clear shift in gene transcripts and MHC protein translation to fast isoforms in the tail, and these shifts are most likely attributed to a drastic increase in levels of thyroid hormones early in development (Butler-Browne and Whalen, 1984; DeNardi et al., 1993). Thyroid hormones play a critical role in muscle development. Notably, studies evaluating development in rodent skeletal muscle have shown that load bearing activity is not essential for fast-contracting muscles to achieve their adult isoform phenotype, which normally consists of predominant expression of the fast MHC-2X and MHC-2B isoforms (Adams et al., 2000). In rats, high levels of thyroid hormones appear to induce the MHC-Neo isoform to shift to fast MHC-2B (DiMaso et al., 2000), whereas low thyroid levels have been found to inhibit or delay the transition to adult fast isoforms (Adams et al., 1999; Butler-Browne and Whalen, 1984; Sugie and Verity, 1985). Relating these previous hormonal findings to *M. domestica*, development beyond the onset of tail prehensility (~5 months), as evidenced by observed distal tail flexion and some grasping/carrying behavior (Fig. 7), demonstrates relative shifting from fast- (MHC-2X and MHC-2B) to slow-contracting (MHC-1β and MHC-2A) isoforms. This shifting pattern is suggested to occur for several reasons: (i) The thyroid gland may

have graded suppressive effects on expression of MHC isoforms in the order of 2X<2A<1 as previously found in mice (Agbulut et al., 2003); (ii) In the absence of load bearing, caudal muscle growth is delayed and this may bias MHC composition in the FCL towards expression of MHC-2A (Adams et al., 2000); and (iii) MHC-1 may not be affected by thyroid hormones in *M. domestica*, thus the primary expression of MHC-1β remains similar to its isoform content at birth, with the observed secondary increases in MHC-1β being more dependent on innervation (Condon et al., 1990) and tail use for nest construction in more mature opossums (Fig. 7).

One of the more curious findings of the present study are mismatches between the mRNA percentage and translation of the protein to MHC isoform expression. The main case in point is the mRNA transcripts for MHC-1β are much higher than those of the other MHC genes across ontogeny, and yet this results in just a modest percentage of MHC-1β protein expression in the FCL (Figs. 4, 5). This might suggest the MHC-1β gene is regulated by a different mechanism compared to the fast/developmental isoforms to where a greater amount of the slow MHC-1β transcripts are required to translate the MHC-1β protein. Alternatively, the mRNA for MHC-1β may be fundamental to translation of the fast MHC isoforms in skeletal muscle. In this scenario, changes in a gene promoter, or modifications to the mRNA for MHC-1β, would result in low transcription of mRNA specific to a fast MHC isoform that is capable of translating relatively high protein expression. A mismatch is also observed for fast MHC-2A, where mRNA increases up to age 5 months and then decreases as the isoform expression continues to increase through adulthood. However, the described mismatches between mRNA and isoform percentage may not uncommon because MHC expression is highly regulated at various levels of control, including transcriptional, translational, and post-translational steps (Andersen and Schiaffino, 1997; Andersen et al., 1999). It is not clear how the exact uncoupling of these different control levels alter the percentage expression of mRNA and its translated MHC isoform, or alter the function of an appendage such as a prehensile tail. Future studies should aim to address mechanistic (molecular) control of skeletal muscle development.

Another factor to be considered is how sex of the animals influences MHC isoform expression. In our previous study of *M. domestica* (Rupert et al., 2014), we reported

retained expression of fast MHC-2B in the FCL of adult males only. This finding is verified by the results of the present study, in addition to retained expression of MHC-Neo in one adult male. Each of these fast isoforms were distinctly absent in adult females. Sexual dimorphic patterns in adult isoform content could be related the functional roles of male and female *M. domestica.* Females may use their tails more frequently while nursing their young, because premature joeys suckle without the insulation of a marsupium and thus, are more sensitive to temperature fluctuations. Increased nest remodeling activity to provide a stable thermoregulatory environment for the young could explain the shift to slower isoform composition in females compared with males. Another possibility is the action of sex hormones influencing MHC content. Some muscles in certain species display sexual dimorphism such as the *m. temporalis* in guinea pigs. The temporalis in male guinea pigs is glycolytic in its metabolism, while that of females is oxidative (Gutmann et al., 1970). Notably, these generalized 'fiber types' could be reversed by castration of the males and/or testosterone injections in the females (Lyons et al., 1986), demonstrating the strong effect sex hormones have on muscle phenotype. In the present study, expression of MHC-Neo was retained up to 7 months in females and one year in male *M. domestica*. This finding was somewhat surprising considering that the onset of tail prehensility was observed at age 5 months. However, expression of developmental isoforms have been found to be lost later in development in muscles that are used for slow, repetitive, and prolonged activity (Agbulut et al., 2003). Considering all of the evidence together, the MHC-neo (and MHC-2B) could be present longer into development in males due to higher testosterone levels coupled with lower frequencies of nest remodeling, whereas sexually mature female opossums would benefit from an earlier development of a slower-contracting, more oxidative fiber type composition in their FCL.

Frequency of use of their semi-prehensile tail was found to be correlated with MHC isoform content. The method by which *M. domestica* uses its tail to collect nesting materials is available (Unger, 1982; Fadem et al., 1986), and data from these previous reports are consistent with our video analysis of tail-specific behaviors for nest construction (Hunsaker and Shupe, 1977). *M. domestica* primarily uses flexion of the distal portion of its tail to grasp and hold nesting material for transport to a nesting site.

Activities related to nest construction serve as part of the behavioral thermoregulatory strategy of the habitually terrestrial opossums (Lisk et al., 1969; Lynch and Possidente, 1978). In the wild, temperature on the rainforest floor changes dramatically between daytime and nighttime hours, and nest remodeling reduces the energetic cost (Fadem et al., 1986) of this environmental pressure. Thus, it is expected that expression of MHC-1β and MHC-2A isoform fiber types would increase with frequency of tail use in wild opossums in response to temperature extremes. MHC content of captive opossums largely match this developmental hypothesis, with the exception of the 7 month old opossums in our age-matched samples. The lack of tail activity quantified for individuals age 7 months could be due to either their activity being performed out of the frame-of-view of the camera, or the constant temperature of 25ºC during data collection. In regards to the latter, another study that evaluated differences in nest construction at different temperatures found that male and female opossums both reliably built nests at lower temperatures (<24ºC), while only females built nests more frequently at higher temperatures (Fadem et al., 1986). These findings agree with that found for percentage nesting activity in 13 month old females in the present study (Table 4). We speculate that wild 7 month old *M. domestica* would use their tail more frequently for grasping/carrying of nesting materials than is observed in this study, especially when rearing young. A final consideration to explain the overall, modest number of total observations of tail-specific behaviors (Table 5) is the small size of the animal enclosures used to house our animals. Increased area and complexity of the enclosure (i.e. enrichment) has been shown to enhance natural activity, including dragging nesting material, in juvenile *M. domestica* (Wilkinson et al., 2010). In that study, only one individual housed in a rodent enclosure used its tail to move nesting material, but the activity was considered to be a repetitive response to stress rather than use for nest construction.

Lastly, changes in body mass with natural growth and development of M. *domestica* also influence MHC isoform shifting in its caudal musculature. Increases in body mass is strongly related to both increases in slow MHC-1β and decreases in fast MHC-2B (Fig. 8). A significant positive correlation coefficient is suggestive of positive allometry for MHC-1β, indicating that the increased expression the slow isoform occurs at a rate faster than normal changes in body size with ontogeny. An increased frequency of nesting with

tail use across ontogeny may be a critical factor influencing this relationship. The significant negative correlation for MHC-2B isoform is suggestive of the opposite trend for its expression during development. Therefore, caudal muscles in larger individuals would be expected to have a slower contracting FCL with few MHC-2B isoform fibers and relatively more MHC-1 fibers, matching what is predicted by body size scaling relationships of MHC fiber shortening velocity (Pellegrino et al., 2003; Toniolo et al., 2007). For the remaining isoforms (MHC-2A, 2X, and Neo), changes in their expression are not dependent (no significant correlation) on body size. Since MHC-2A expression was positively correlated with the frequency of tail grasping/carrying of nesting materials, we can deduce that tail use is a better predictor of percentages of MHC-2A content. Again, the superimposition of more frequent use of tail for nest construction on development must be interpreted as a functional shift in MHC expression (Figs. 7, 8). Higher percentages of MHC-1β and 2A isoform fibers are appropriate for grasping tasks requiring repetitive, low force contractions, while the properties of fast MHC-2X and 2B fibers do not match these functional demands and therefore, expression of these isoform fibers decrease with tail use or development.

Evolutionary implications and Conclusions

As an extension of our previous work with caudal muscle in the Virginia opossum (*Didelphis virginiana*: Hazimihalis et al., 2013) and woolly opossum (*Caluromys derbianus*: Rupert et al., 2014), the ontogeny of MHC isoform expression in the tail of *M. domestica* offers further insight into specialization of prehensile tails of didelphid marsupials. Modern opossums descend from a common South American arboreal ancestor (Cozzoul et al., 2006; Szalay, 1994). The woolly opossum serves as a modern representation of the ancestral condition in this lineage. *C. derbianus* is strictly arboreal and uses its fully prehensile tail as a fifth appendage primarily for arboreal locomotor maneuvering (Youlatos, 2008; Schmitt and Lemelin, 2002; Dalloz et al., 2012). The FCL of *C. derbianus* showed larger expression of the fast MHC-2A and MHC-2X isoforms in comparison to *M. domestica* to provide high force to suspend its body mass. Percentages of MHC isoform content from 13 month old individuals in the present study are consistent with those reported for adult *M. domestica* by Rupert et al. (2014), and further indicate that expression of MHC-2B in adults may likely be an ancestral retention. A

similar evolutionary hypothesis was generated from fiber typing studies of the perivertebral muscles in Monodelphis and other small therian mammals (Schilling, 2009). In particular, selective pressures acting to change the functional role of these muscles to that of stabilizers consequently caused a fast-to-slow shift in the base expression of MHC. The gene MYH4 (Table 1) is functional in *M. domestica*, but is down regulated to where it no longer translates a functional MHC-2B isoform as the role of the FCL changes across ontogeny. Our findings further suggest that fast isoform expression may be ancestral for prehensile-tailed mammals.

Corresponding with diversification in opossums, opossums became adapted for different habitats. Like short-tailed opossums, *D. virginiana* adopted a terrestrial locomotor habit on the forest floor (McManus, 1970; Hunsaker, 1977) and the prehensility of its tail was reduced to semi-prehensile function (Emmons and Gentry, 1983). Both species primarily use their tail for the adaptive behavior of carry materials for nest building (Layne, 1951), and they share a fiber type distribution in their FCL that includes similar percentages of slow MHC-1β fibers as adults (Hazimihalis et al., 2013). Interestingly, *D. virginiana* has the ability to suspend its body from the tail when young, but loses that ability as an adult (McManus, 1970). Comparable patterns of changes in the degree of prehensility across ontogeny are also observed in squirrel monkeys (Genus: *Saimiri, Cebidae*) (Thorington, 1968; Rosenblum, 1968). This is in contrast to *M. domestica* that is habitually terrestrial with a short tail, and never has the ability for suspension. While the exact mechanism of developmental changes in prehensile ability is not known, we speculate that a combination of alterations in MHC gene regulation and rapid increases in body mass in *Didelphis* and *Saimiri* explain the available observations. Considering our data from *M. domestica* as a model, where the primary expression of MHC in caudal flexor musculature is fast. A distribution of fast (and larger) MHC isoform fiber types would provide the high force required for suspension at a young age. However, transitions in expression to slower MHC isoform composition in the tail of other taxa may be coded by gene regulation to occur at an earlier critical growth period (<5–7 months) than was observed in *M. domestica*. It is also possible that the increases in body mass are triggered by hormones during this period, resulting in a natural fast-to-slow transition in MHC isoform expression corresponding with changes in body size.

Moreover, the tail structure may be too weak to support the body mass of *Didelphis* and *Saimiri* beyond a certain critical mass (both species exceed 1 kg as adults). These possibilities need to be explored to improve understanding the developmental processes leading to tail prehension.

Overall, the outcomes of this study suggest that transitions in MHC expression in a semi-prehensile tail are different than those in the limbs of similarly-sized therian mammals. Weight bearing (on limbs) provides a stimulus for developing muscle fibers expressing an overall faster MHC isoform composition. In contrast, using data from our present and previous studies, we may now conclude that MHC-2A is the isoform of primary expression in the tails of didelphid marsupials. The flexor tail musculature of a habitually terrestrial species becomes notably slower-contracting by selective up regulated expression of slow MHC-1β and consequent down regulation of fast MHC-2B with growth and development. Retained expression of MHC-2B and developmental isoform MHC-Neo late into development, however, indicates that there is a critical growth period is between 7 and 13 months. At this time, *M. domestica* is sexually mature and appears to have full grasping capability with its tail, and the increased expression MHC-1β and 2A are dependent on increased use of its tail for nest construction as adults. Thus, ontogenetic shifts from faster- to slower-contracting isoforms may be associated with muscle 'tuning' for the repetitive nest remodeling tasks requiring sustained, low force contractions of the caudal flexors. Indeed, the properties of MHC-1β and 2A isoform fibers match well with these functional requirements. Finally, while the genomic results presented here support the ontogenetic changes in the MHC proteome, additional work is required to explain the high degree of posttranscriptional regulation of MHC-1β and mechanisms of how mRNA becomes translated into the functional slow isoform protein in mammalian skeletal muscle.

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APPENDIX

INTRODUCTION

This study will investigate how the myosin heavy chain (MHC) composition of skeletal muscle changes with the ontogeny of prehensile tail function. The grey short-tailed opossum (*Monodelphis domestica*) will serve as model species to study the early stages of mammalian muscle development because the offspring of this didelphid marsupial are more immature than any non-precocial eutherian mammal at birth (Barthelemy and Cabana, 2005). Moreover, didelphids are considered the best available group for comparison with eutherian mammals because they have a basic body plan closer to that of the common ancestor of eutherians than their Australian marsupial relatives, which have been reproductively isolated from the other generalist eutherian species for over 70 million years (Kimble, 1997). Specifically, the development of the musculature in their semi-prehensile tails provides an opportunity to evaluate changes in tail muscle physiology and function from early development to adulthood. At birth, the tail has no function and is dragged for the first 6 weeks before progressively developing prehensile capabilities to be used for carrying nesting material and for nest construction/remodeling (Pflieger et al., 1996; Macrini, 2004). To identify the timing of the observed ontological changes in tail use, MHC isoform composition will be determined in the tail of *M. domestica* from age 12 weeks to late adulthood. Herein, use of *M. domestica* as a model to answer questions about muscle fiber ontogeny and use in prehensile tails will be developed through a discussion of their speciation, ecology, behavior, development, and morphology and fiber type distribution of their semi-prehensile tail.

Origin and Speciation

Regions rich in resources such as the rainforests of South America can provide an environment for increased species diversity (Hortal, 2008). Didelphid marsupials (opossums) are a prime example of mammalian evolution, diversity, and specialization in these neotropical regions. The appearance of marsupials originated in the Cretaceous-Tertiary Boundary when marsupials and placental mammals (eutherians) diverged from a common mammalian ancestor (Archibald, 1982; Case and Woodburne, 1986; Caroll, 1997). Marsupials of the order Didelphimorphia emerged and have evolved into the large family Didelphidae (and four subfamilies) of opossums inhabiting the Americas,

representing 19 genera with more than 100 species (Voss and Jansa, 2009), and ranging from the southern-most regions of Argentina to southern Canada (Hershkovitz, 1997; Nowak, 1999). Didelphids are native to southern South America and represent the largest order of marsupials in the western hemisphere. Overall, the climatic conditions and abundance of resources found in South America allowed for broad speciation (over 85 currently recognized species in the largest sub-family), and most genera became successful by utilizing the natural resources and a multitude of available niche space (Hershkovitz, 1997; Nowak, 1999).

The genus Monodelphis is the most diverse of the Didelphidae family (subfamily: Didelphinae; Tribe: Marmosini), comprising 21 recognized species (Voss and Jansa, 2009) of relatively small terrestrial opossums with short tails, small ears, and variable pelage patterns (Gardner et al., 2005; Pine and Handley, 2008; Solari, 2010; Vilela et al., 2010). The species *Monodelphis domestica* derives its name from the Greek word *monodelphis* meaning "single womb" referring to the lack of a marsupium (pouch), and *domestica*, which is Latin for "domestic" referring to the habit of entering human dwellings (Braun and Mares, 1995). Within the genus Monodelphis, there are some subtle differences between *M. domestica* and its closest relatives. For example, *M. domestica* differs from *M. maraxina* by having more tail bristles and more well-developed auditory bullae (Pine, 1979). *M. domestica* is also larger, and has a more developed sagittal crest than *M. kunsi* (Anderson, 1982). In addition, *M. domestica* is different from nine other species in the genus by its fur color that lacks red, reddish-brown, or orange hairs (as seen in *M. dimidiate, M. brevicaudata, M. henseli, M. scallops, M. emiliae, and M. sorex*), or by lacking dorsal stripes (e.g., *M. americana, M. iheringi, and M. unistriata*) (Thomas, 1888; Peterson and Pine, 1982; Macrini, 2004).

Ecology and Morphology

M. domestica can be found in the southern rim of Amazon Basin in eastern, southern, and central Brazil; eastern Bolivia; and northern Paraguay and Argentina (Myers and Wetzel, 1979; Streilein, 1982; Emmons and Feer, 1990; Redford and Eisenberg, 1992; Eisenberg and Redford, 1999). It is a solitary, habitually terrestrial opossum that spends most of its time on the rainforest floor and uses low thorn scrub, high thorn scrub, cultured and abandoned fields, and granitic outcrops as common habitats. For a small opossum, its

home range is quite large, with little overlap of individuals inhabiting the same space. For example, in the Caatinga region of Brazil, the average home range for adult male *M. domestica* is 1209.4 m^2 and 1788.8 m^2 for adult females. It has also been noted that the population size increases in the Caatinga during the dry months (Streilein, 1982). In that same study, each home range had a low population density of only 0–4 adults per range (Streilein, 1982). Home ranges were quantified by markings made by the side of the head rubbed against forest floor material. Males may also rub the area around the nest entrance with their abdomen to drag their scrotum to mark their "personal space".

Body mass of adult *M. domestica* ranges between 58–95 g with a mean body mass of 71.4 g (Redford and Eisenberg, 1992). Males usually have greater mass than females because females invest more energy in reproduction than males, and growth rates are reduced in females after their first litter (Bergallo and Cerqueira, 1994). The face and dorsal pelage are a grayish brown hue, while the cheek pouches are gray with a slight yellow tint, and the mandibular region and ventrum are a lighter gray with an orange tint (Thomas, 1888; Emmon and Feer, 1990; Redford and Eisenberg, 1992). Notably, *M. domestica* lacks a marsupium and has a short, semi-prehensile tail. The tail is furry for the first 1–2 cm at its base and is hairless for the remainder of its length (Emmons and Feer, 1990). In male *M. domestica*, the average tail length is 8 cm, which is approximately half the length as the head and body (total body length: ~15 cm), giving this species a relatively low tail length-to-body length ratio (i.e., relative tail length) of nearly 0.5 (Rupert et al., 2014). However, this ratio is typically lower for female *M. domestica* as they display a slightly shorter tail length (7 cm) on average than males (Fadem and Rayve, 1985; Macrini, 2004; Rupert et al., 2014).

a. Semi-prehensile tail

Prehensile tails have evolved independently 14 times among 40 extant mammalian genera (Emmons and Gentry, 1983; Emmons and Feer, 1990; Organ, 2010). Mammals with prehensile tails include didelphid and most phalangerid marsupials, anteaters, pangolins, some rodents, and a few non-human primates (e.g., *Ateles* and *Cebus*) and carnivorans (e.g., *Potos*) (Youlatos, 2002; Organ et al., 2009). A fully prehensile tail has been defined as a tail that can alone support the weight of the suspended body, while a semi-prehensile tail is capable of supporting only a portion of the body weight (Emmons

and Gentry, 1983). Fully prehensile tails are found in arboreal species that rely on their tail to act as a fifth appendage for balance and stability in arboreal locomotion. The tail is used during specialized arboreal maneuvers such as bridging and cantilevering, or to suspend the body mass by tail hanging and foot hanging (Lemelin et. al., 2003; Youlatos, 2008). A semi-prehensile tail, as found in *M. domestica*, is primarily used for adaptive behaviors involving lifting and carrying nesting materials and their offspring (Grizimek, 1990; Nowak, 1991). The semi-prehensile tail of *M. domestica* is also highly instrumental in building and remodeling nests (see below), where the tail can be used to tightly weave nesting debris, and their nest may be remodeled several times per day (Unger, 1982; Macrini, 2004).

Differences in tail use between arboreal (and scansorial) and terrestrial opossums are apparent in the morphology of the caudal vertebrae. For example, the fully prehensile tail of the highly arboreal species *Caluromys derbianus* has 37 caudal vertebrae (Hall and Kelson, 1952) compared to the semi-prehensile tail of *M. domestica* containing 19–20 caudal vertebrae (Pridmore, 1992) (also see Fig. 1: Rupert et al., 2014). The tail can be segmented into three distinct sections based on the caudal vertebrae: proximal, transitional, and distal. In general, prehensile-tailed mammals tend to have longer proximal region of the tail than nonprehensile-tailed species (Organ and Lemelin, 2011). The proximal region of the tail possesses ventral and neural arches, a pair of transverse processes, and articulate with one another by zygapophyseal joints. The neural arches are higher in prehensile tails to permit the passage of spinal nerves for increased innervation to the tail musculature (Ankel, 1965, 1972; Grand, 1977). The zygapophyseal joints articulate, allowing for flexibility in the sagittal plane while restricting movement in other planes (Shapiro, 1993, 1995; Ward, 1993). Further caudally in the proximal tail region, the neural arches, spinous processes, and transverse processes become smaller until reaching the last proximal vertebrae, known a the transition vertebrae (TV) (Ankel, 1962). The TV can be identified by possessing zygapophyseal joints on its proximal end, while lacking the zygapophyses on its distal end. The transitional tail region spans from the TV, with each successive vertebrae increasing in craniocaudal length (CCL) until reaching the longest vertebrae (LV), which mark the end of the relative short transitional tail region. All remaining vertebrae after the LV decrease in CCL and are part of the

distal tail region (Russo and Young, 2011). Distal tail vertebrae are characterized by having longer, more rounded vertebrae than in the proximal tail region. These vertebrae also have two reduced pairs of transverse processes, lack zygapophyses, and articulate only through intervertebral discs, which allows for greater ranges of intervertebral motion in all planes of movement. Lastly, hemal processes (attachment sites for the primary tail flexor muscles) of caudal vertebrae in prehensile tails are more well-developed than those of caudal vertebrae in non-prehensile tails, and can distinguish prehensility better than traditionally used external measurements (Organ, 2010). The size of the hemal processes progressively decreases along the length of the tail and hemal processes are the attachment sites for flexor musculature that causes tail flexion (German 1982, Organ, 2007, and Organ, 2010) during grasping tasks. Like the prehensile tails of non-human primates, didelphids have been observed to have hemal processes spanning the entire tail length. However, if chevron bones (i.e., hemal arches) are present, they tend to be restricted to the more proximal portions of the tail, and this is consistent across all tail types (Organ, 2010).

b. Tail myology

All members of Didelphidae have the same caudal muscles, but the robustness of musculature and length of the muscle segments vary between prehensile tail types. *M. domestica* has two main tendons that originate on the superior face of the sacrum and insert on the distal-most caudal vertebrae (Rupert et al., 2014). These two tendons are evenly placed along the dorsal side of the tail and are superficial to the main tail extensor, *m. extensor caudae longus* (ECL) which is located along the dorsal aspects of each caudal vertebrae. There are also two complimentary tendons on each side of the tail that originate laterally at the base of the tail and span the entire tail length to attach to the lateral aspects of the caudal vertebrae. These tendons separate the inferior surface of the ECL from the superior surface of the main tail flexor, *m. flexor caudae longus* (FCL). These tendons meet at the distal-most caudal vertebrae to form one common tendon sheath at the tail tip (Rupert et al., 2014).

Including the ECL and FCL, there are five caudal muscles that each Didelphidae species have in common. On the dorsal side of the tail, *m. caudofemoralis* (CF) and ECL are the two extensor muscles (Kirsch, 1973). The CF originates from both the greater

trochanter of the femur and the posterior superior iliac spine to insert on the dorsolateral aspects of the proximal caudal vertebrae. Although it is considered a caudal muscle, the main action of CF is hindlimb abduction with a very limited role in tail extension (Kirsch, 1973). The ECL originates on the superior face of the sacrum and spans the entire length of the dorsal tail, traversing and attaching to the superior surfaces of the transverse processes of each caudal vertebra to ultimately insert on the dorsal surface of the most distal caudal vertebrae. The distal-most insertion of the ECL muscle becomes tendinous at the tip of the tail (Rupert et al., 2014). On the ventral side of the tail, *m. flexor caudae brevis* (FCB) and FCL are two superficial flexor muscles, while *m. intertransversari* (IT) are the deep flexors that span between each caudal vertebra. FCB is located at the proximal end of the tail and is situated deep to the CF and superficial to the FCL. It originates on the posterior portion of the iliac fossa and inserts on the ventrolateral faces of the proximal caudal vertebrae (most distal insertion is where the hairless portion of tail begins with respect to surface anatomy) (Kirsch, 1973; Rupert et al., 2014). The FCB also inserts into a 'flexor tendon' along the ventral side of the tail, giving this relatively short bellied muscle a long moment arm for facilitating tail flexion with the FCL. The FCL is a segmented muscle that originates on the inferior surface of the sacrum and spans the entire length of the ventral tail, traversing and attaching to the inferior surfaces of the transverse processes of each caudal vertebra, to ultimately insert on the ventral surface of the distal-most caudal vertebrae (Kirsch, 1973; Rupert et al., 2014). Like the ECL, FCL also becomes tendinous at the tip of the tail. Lastly, the intertransversari group are unique muscles, the origin of each IT muscle is located on the caudal aspect of each transverse process and has an insertion onto the proximal aspect of the transverse process of the next sequential vertebra (Organ, 2010). The IT muscles have an important role in the prehensile ability of the tail as contraction of IT allows for flexion at each vertebral segment articulation, thus helping the tail coil around substrates (Organ et al., 2009). To enable the tail to coil around a substrate quickly requires expression of fast-contracting muscle fibers in the caudal musculature, while use of the tail for postural and adaptive behaviors would require prolonged contractions at low velocity, and thus expression of slow-contracting fiber types.

c. Myosin heavy chain isoforms

Identification of specific MHC isoforms is important because these protein subunits directly determine the contractile properties of muscle fibers and by extension, help to determine the contractile performance of entire muscles (Sciote and Rowlerson, 1998; Zhong, 2001; Kohn et al., 2011; Sokoloff, 2010; Schiaffino and Reggiani, 2011). Three techniques are frequently used to properly identify individual MHC isoforms (and fiber types): Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Immuno-Blotting (Western Blot), and immunohistochemistry (IHC). Analysis with SDS-PAGE uses electrophoresis to separate MHC isoforms on a polyacrylamide gel based on their molecular weight, with the lighter isoforms migrating through the gel farther than the heavier isoforms (Talmadge and Roy, 1993; Mizunoya et al., 2008). Western Blot analysis enables the MHC isoforms separated by SDS-PAGE to be transferred onto a reactive (PVDF) membrane that is subsequently incubated with monoclonal antibodies (mAbs) specific to individual MHC isoforms, allowing a precise identification (by reaction against mAbs) of each individual isoform band (Kohn et al., 2007, 2011). IHC is performed the same as Western Blot except frozen serial sections of muscle tissue (mounted to microscope slides) are reacted against mAbs to confirm MHC isoform fiber type. The IHC technique is important for identification of hybrid fibers that express more the one MHC isoform (Hazimihalis et al., 2013; Rupert et al., 2014) and allows for fiber types to quantified in an entire cross-section of a muscle.

Specifically, the MHC isoforms expressed within a muscle regulate the rate at which ATP is hydrolyzed by the myosin ATPase (i.e., myosin heads), and thus determine the maximum shortening velocity (V_{max}) and power output (W) of a muscle fiber (Rowlerson, 1994; Schiaffino and Reggiani, 1996, 2011). There are four conventional MHC isoforms that are expressed in mammalian skeletal muscles (listed in order of the lowest-to-highest V_{max}): MHC-1, MHC-2A, MHC-2X, and MHC-2B (Toniolo et al., 2004, 2005, 2007; Kohn et al., 2007, 2011; Zhong, et al., 2001; Zhang et al., 2010). The four fiber types (by both their metabolic properties and myosin ATPase rate) correspond to MHC muscle fibers expressing the specific isoforms of the same name. MHC-1 isoform fibers isoforms are slow, oxidative and produce low force that can be sustained without fatigue. MHC-2A isoform fibers are fast, highly oxidative and produce higher

force and power than MHC-1 fibers. MHC-2X isoform fibers are fast, moderately oxidative/glycolytic and produce higher force and power than MHC-2A fibers. Lastly, MHC-2B isoform fibers are fast, highly glycolytic and produce the highest amount of force and power of any fiber type, but fatigue easily (Schiaffino and Reggiani, 1996; Sciote and Rowlerson, 1998; Zhong et al., 2001; Kohn et al., 2011). Therefore, the more oxidative fiber types (MHC-1 and MHC-2A) can produce force and power over a long duration, whereas more glycolytic fiber types (MHC-2X and MHC-2B) can produce high force and power for short durations.

Marsupials have been shown to express each of these four MHC isoforms in their limb muscles (Lucas et al., 2000; Zhong et al., 2001). This finding is significant as marsupials do not appear to show the same body size dependence on MHC isoform expression as observed in eutherian mammals (Seow and Ford, 1991; Pellegrino et al., 2003; Marx et al., 2006). Only relatively small eutherian mammals, including most members of the order Rodentia and family Leporidae, express the fast MHC-2B isoform in their skeletal muscles (Bottinelli and Reggiani, 1991; Galler et al., 1997; Oleg, 2004). However, MHC-2B is expressed in extraocular muscles of numerous mammals regardless of body size (Toniolo et al., 2005, 2007; Rhee and Hoh, 2008; McLoon, 2011). With the exception of these specialized fast muscles, the general rule of muscle scaling is that as mammals increase in body size their muscles become slower contracting and less powerful (Rome et al., 1990; Seow and Ford, 1991; Pellegrino et al., 2003; Marx et al., 2006; Toniolo et al., 2007). Terrestrial didelphids (e.g., *Didelphis* and *Monodelphis*) can only walk and trot at relatively slow speeds (Schmitt and Lemelin, 2002; Youlatos, 2008; Butcher et al., 2011) and at this time, it is not clear if the South American forms of marsupials also express pure fast MHC-2B fibers in their limb muscles (Peters et al., 1984; Sciote and Rowlerson, 1998).

Few studies have investigated fiber types and MHC isoform composition in the prehensile tails of opossums (Hansen et al., 1987; Hazimihalis et al., 2013; Rupert et al., 2014). Previous studies investigating MHC isoform expression in the caudal muscles of terrestrial opossums (Hazimihalis et al., 2013; Rupert et al., 2014), identified an abundance (~75%) of fast, oxidative MHC-2A/X hybrid fibers in the FCL of *Didelphis virginiana* and *M. domestica*. Specifically, the distribution of MHC isoform fiber types in

the FCL of *M. domestica* was reported to be 17.0% MHC-1, 1.3% 1/2A, 9.0% 2A, 75.2% 2A/X, and 0.3% 2X/B hybrid fibers (Rupert et al., 2014). These data provide important details about the sub-divisions of fast MHC fiber types needed to more accurately interpret tail function. Notably in *M. domestica*, a relatively large percentage of slow MHC-1 fibers, in addition to pure fast, highly oxidative MHC-2A fibers, were observed. These two fiber types may be important for the repetitive nest remodeling tasks of *M. domestica.* The expression of the MHC-2B isoform was only observed in males and restricted to the proximal tail region (Rupert et al., 2014). The explanation for the expression of fast MHC-2B fibers is incomplete, but they are believed to be an ontological retention from early in development that continue to be expressed in males, which have a reduced role in nest construction and remodeling (Rupert et al., 2014).

Aside from the MHC isoforms typically expressed in skeletal muscles, there are also a few specialized isoforms that also may be present. The first is MHC-L (laryngeal isoform) found in the thyroarytenoid and lateral cricoarytenoid muscles, and these isoform fiber types have been reported to be very resistant to fatigue with a high SDH (i.e., oxidative) activity (Hoh 2005; Mascarello and Veggetti 1979). The second is MHC-M (masticatory isoform) which is only found in masticatory muscles (e.g., masseter and temporalis). Carnivores, primates (except humans), some species of bats, and a number of marsupials have this jaw-specific MHC isoform (Rowlerson et al., 1983). MHC-M isoform fibers are fast, produce high force, and have moderate shortening velocity that is ideal for high power tearing and chewing (Toniolo et al., 2008). The third, and last group of the specialized isoforms, are developmental in nature and include MHC-emb (embryonic) and MHC-neo (neonatal). These developmental isoforms are formed during a prenatal developmental when essentially no load (i.e., stress and strain) is placed on muscles. It has been suggested that load-bearing could be a physiological stimulus for the transition of the embryonic and neonatal isoforms into adult MHC isoforms during the load-bearing postnatal phase of life (Caplan et al., 1983). The prehensile tail muscles found in *M. domestica* may also express these developmental MHC isoforms as juveniles. However, expression of MHC-neo varies with age and its migratory position in SDS-PAGE gels has been shown to be inconsistent among species tested previously (Toniolo et al., 2005, 2007).

Behavior and Development

Due to their solitary nature, both male and female *M. domestica* build their own nests to ensure survival in the rainforest. These nests are constructed using the variety of objects found in their home range, including leaves, grasses, bark, snake skin, paper, plastic, and cloth. While both sexes construct their own nest, the females tend to construct more complex and tightly interwoven structures (Macrini, 2004). The nesting material is initially collected using the mouth and is then manipulated between the forelimbs and hindlimbs to their semi-prehensile tail. More specifically, *M. domestica* takes material with the mouth and tucks it into its forepaws. The nesting material is then pushed from the forepaws, along the underside of the body, where it is moved with shuffling motions of the hindlimbs to the semi-prehensile tail. The tail then grabs the material tightly by coiling around it. After several sequences of moving material from the mouth-to-tail, *M. domestica* carries the collected material in the coiled tail to the nest for construction (Unger, 1982; Fadem et al., 1986). Interestingly, the motion of transferring nesting material from the mouth to their semi-prehensile tail also serves a reproductive function. It has been hypothesized that the described actions "odor marks" the material by rubbing it across the sternal gland is the male marking his territory (Unger, 1982). This is important for *M. domestica* because the females do not show an external estrous cycle. Isolated females remain anestrus, but are induced to estrus within 4–11 days after exposure to male pheromones (Fadem, 1987; Harder et al., 1993).

It has been observed that while in the nest, *M. domestica* sleeps on its side and curls tightly during rainy or cold weather. The sleeping posture is more open during warmer weather (Macrini, 2004). Nest building in these mammals serves two biological functions: 1.) protection from environmental temperature extremes, and 2.) safety during the bearing and raising of the young (Lisk et al., 1969; Lynch and Possidente, 1978). Being an efficient nest builder is especially important for opossums because marsupials have a basal metabolism approximately 30% lower than that of eutherian mammals (Dawson and Hulbert, 1969). Therefore, nest-building is a thermoregulatory behavior, which is enhanced at low temperatures and reduced at high temperatures (Sealander, 1852). As the temperature changes in the rainforest, *M. domestica* spends a lot of its time

remodeling the nest to accommodate the environmental temperature fluctuations (Macrini, 2004).

The development of *M. domestica* is especially important when studying changes in muscle structure and function because opossums are born severely underdeveloped and undergo a relatively long postnatal development (Pflieger et al., 1996). A newborn *M. domestica* is only able to use its forelimbs for movement, while the hindlimb buds passively follow trunk undulations (lateral flexion motions). During the fifth and sixth week of development, opossums are able to bear weight on all four limbs and their tail begins to be lifted and maintained in line with the body axis during locomotion. At seven weeks, immature opossums are largely similar to adults (except for absolute body size) with comparable locomotor function, although young opossums typically walk/run at relatively higher speeds than their adult counterparts. *M. domestica* reaches the adult body size around the ages of 5–7 months, which corresponds with their sexual maturity. By age seven months, *M. domestica* have full use of their semi-prehensile tail and they exhibit routine nest construction (Pflieger et al., 1996). While use of the tail for nest building tasks has been documented, it is more difficult to determine if their tail is also postural and important for maintaining locomotor balance during their development and into adulthood. This is because adult opossums that lose their tail do not show notable locomotor motor deficits (Pflieger et al., 1996).

a. Fiber type shifting (MHC isoform changes with development)

Many mammals are born with highly underdeveloped skeletal muscles and important changes in muscle fiber type occur early in postnatal development. However, there is relatively little data on changes in MHC isoform fiber type during postnatal development and very little work on marsupial model systems. Moreover, even less is understood about caudal muscle development (Ovalle, 1976). Surprisingly, no previous studies, have investigated muscle development and fiber type changes in the common laboratory marsupial, *M. domestica,* but several studies investigating the role of neural and hormonal cues, and mechanical load alterations during postnatal development, have been performed using the mouse and rat (Schiaffino and Reggiani, 2011). During the first few weeks of development in mice and rats, changes occur that cause a transition in muscle fiber type composition. Two important observations are 1.) the progressive disappearance

of embryonic and neonatal MHC isoforms that appear to be lost more slowly in the MHC-2A fibers developed in the adult stages (Schiaffino et al., 1988), and 2.) the up-regulation and expression of adult fast MHC genes for 2A, 2X, and 2B isoform fibers (DeNardi al et., 1993).

In particular, the rat soleus muscle has been used as a common model for developmental muscle studies. A neonatal rat has a soleus containing only 50% slow MHC-1 fibers compared with 80% slow MHC-1 fibers in adult rats. During development, this muscle undergoes an increased distribution of slow MHC-1 fibers concurrent with a down-regulation of embryonic and neonatal MHC expression (Butler-Browne and Whalen, 1984). The increase in slow fibers is due to a progressive transition of the fast MHC-2A isoform into MHC-1 isoform fibers (Butler-Browne and Whalen, 1984). Another reported observation in rat (and mouse) hindlimb muscles is a shift from embryonic/neonatal isoforms to adult fast MHC isoforms during the first 2–3 weeks of postnatal development. In fact, during the first 2–5 days after birth, transcripts for MHC-2A, 2X, and 2B are detected in the limb muscles and display expression patterns corresponding to where these myosins later appear in the adult muscles. For example, in the tibialis anterior muscle, MHC-2B isoforms are expressed in the superficial region of the adult muscle, while the MHC-2A isoforms are found only in deep region (DeNardi al et., 1993). Another example are the fast limb muscles of mice that contain a large distribution of MHC-1 fibers during the first couple weeks after birth, but then later transition into fast MHC-2A, 2X and 2B isoform fibers, resulting in the disappearance of slow fibers in their limb muscles (Whalen et al., 1984; Agbulut et al., 2003). An important factor in this transition may be the mechanical load that is placed on the neonatal fibers after leaving the intrauterine environment, thus causing them to transition into the postnatal, adult fiber types that are required for weight-bearing activity (Caplan et al., 1983). A final factor to consider is the level of hormone produced by the thyroid gland, which is low until birth, but then increases to a maximum level 2–3 weeks postnatal (Gambke et al., 1983). A possible link between muscle fiber type transition and thyroid hormone level is suggested by the finding that the change from neonatal-to-adult fast MHC isoforms is delayed by hypothyroidism and accelerated by hyperthyroidism (d'Albis, et al., 1990; Gambke et al., 1983).

Objectives and Hypotheses

The main objective of this study is to evaluate the ontogeny of MHC isoform expression in the tail musculature of *M. domestica* from age 12 weeks to late adulthood. How MHC composition changes in the semi-prehensile tail of *M. domestica* as it develops will improve our understanding of adult MHC isoform expression as it relates to the timing of prehensility and tail use for nest building tasks in didelphids. This study builds on previous work quantifying MHC isoform fiber type distributions in the semi-prehensile tails of adult terrestrial opossums *D. virginiana* (Hazimihalis et al., 2013) and *M. domestica* (Rupert et al., 2014). It is hypothesized that juvenile stages of development (3–7 months) in *M. domestica* will be characterized by a predominant expression of fast MHC isoforms that will transition to slower MHC isoform fiber types as development progresses to adulthood (≥ 1 year old). Specifically, it is predicted that: (1) juveniles will express developmental isoforms, namely neonatal (MHC-neo), in addition to a large percentage of the fast skeletal isoforms MHC-2X and 2B, throughout the tail, (2) at approximately 7 months; isoform expression will shift to an abundance of MHC-1, 2A, and 2X isoforms; corresponding with the onset of nest building and remodeling behavior, and (3) males will retain the expression of fast MHC-2B fibers through adulthood because of their reduced roles in nest construction and care for young opossums early in life.

METHODS AND MATERIALS

Animals

Five (*N*=5) immature and six (*N*=6) adult gray short-tailed opossum (*Monodelphis domestica*), will be used in this study. Five adults, age 1 year old, are from a previous study (Rupert et al., 2014) and the MHC isoform data from these individuals will be used here for comparative analysis. Male and female *M. domestica* were obtained in collaboration with the Texas Biomedical Research Facility in San Antonio, Texas. Animals were euthanized by CO_2 asphyxiation followed by cervical vertebral dislocation, and upon confirmation of death, fresh caudal muscle tissue was harvested from the tails and shipped frozen (on dry ice) to Youngstown State University. All experimental procedures followed protocols approved by the Youngstown State University Animal Care and Use Committee (YSU IACUC: 01-12 and 04-12).

Supplemental Table 1. Morphometric data for *M. domestica* used in this study.

Opossum	Sex	Age	Body mass (g)	Hallux length (cm)	Head-Body length (cm)	Tail length (cm)	Relative Tail Length
Juvenile							
Md 3	M	3 mo	58.9	0.4	13.5	7.5	0.55
Md 4	M	4 mo	92.1	0.5	14.5	8.0	0.55
Md 5	M	5 mo	104.2	0.6	17	8.0	0.47
Md 6	M	6 mo	117.0	--	17	10.0	0.59
Md 7	F	7 mo	72.7	0.5	14.5	7.5	0.52
			89.0±23.4	**0.5±0.8**	**15.3±1.6**	**8.2±1.04**	**0.54±0.04**
Adult							
Op1*	M	1 yr	126.0	0.5	17.0	9.5	0.56
Op2*	F	1 yr	76.6	0.5	14.0	7.5	0.54
Op3*	F	1 yr	83.3	0.4	15.5	7.5	0.48
Op4*	F	1 yr	86.5	0.4	15.3	7.2	0.47
Op5*	M	1 yr	131.8	0.5	16.2	8.3	0.51
Op6	M	2 yr	136	0.5	16	9	0.56
			101±25.9	**0.5±0.05**	**15.6±1.1**	**8.0±0.9**	**0.5±0.04**

*Individuals used in our previous study (see Rupert et al., 2014)
Values in bold are mean (± s.d.)
Relative tail length is calculated as tail length divided by head-body length

Muscle Tissue Preparation

Caudal muscle tissue was sampled from the tails immediately post-mortem. Briefly, the tails were divided into 'proximal', 'transitional', and 'distal' regions for muscle dissection (Hazimihalis et al., 2013; Rupert et al., 2014). Tissue blocks from the FCL muscle from each tail region were harvested (stored at -80°C) and prepared for electrophoresis by the following method: directly freezing the muscle blocks in liquid nitrogen, grinding them to powder, homogenizing 50 mg of muscle powder in 800 µl (ratio 1:16) of Laemmli buffer with 62.5 mM Tris (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, and 2.3% SDS (Laemmli, 1970), and centrifugation of the muscle homogenates at 13k rpm for 10 min. After centrifugation, the stock supernatant was decanted into sterile microcentrifuge tubes and used to make the protein samples for gel loading. Protein samples were diluted (1:500) to a final protein concentration of ~0.125 µg/µl with gel sample buffer containing 80 mM Tris (pH 6.8), 21.5% glycerol, 50 mM DTT, 2.0% SDS, and 0.1% bromophenol blue (Mizunoya et al., 2008). Samples were then heated (90ºC) for 5 min, and either loaded in gels or stored at -20°C until analysis.

SDS-PAGE and Densitometry

MHC isoforms will be separated using established methods (Talmadge and Roy, 1993) performed with slight modifications (Mizunoya et al., 2008; Rupert et al., 2014). The acrylamide-*N,N'*–methylenebisacrylamide (Bis) ratio of the gels will be 50:1, with the total acrylamide percentage equaling 8% and 4% in the separating gel (35% glycerol) and stacking gel (30% glycerol), respectively. The basic formulation of the electrode buffer will be 50 mM Tris (pH 8.3), 75 mM glycine, and 0.5% SDS; upper buffer 6X the concentration of the lower buffer and also contained 0.12% β-mercaptoethanol (Mizunoya et al., 2008). Approximately 1.0 μg of protein will be loaded per gel lane, and electrophoresis will be run on a mini-PROTEAN Tetra system (Bio-Rad, Hercules, CA USA) at constant low voltage (140 V) for 21–24 h at 4°C (Talmadge and Roy, 1993; Mizunoya et al., 2008). Gels will be stained with silver for visualization of MHC isoforms and imaged using a Pharos FX Plus system (Quantity One software: Bio-Rad) having a resolution to 50 μm. MHC isoform identity will be evaluated by band resolution, comparative migration patterns, and density of the myosin protein bands. MHC isoform protein content will be quantified by densitometry in Image J (v.1.43: NIH) using the brightness area product (BAP) method of Toniolo et al. (2008). In each gel lane (representing a tail region of one individual), band intensity values will be summed and then used to calculate a percentage for each MHC isoform. Percent MHC isoform composition will be averaged across 2–3 independent densitometric quantifications made from separate gel experiments.

Western Blotting

Companion SDS-PAGE gels will be run with higher amounts of protein (10–15 μg) loaded per gel lane for western blot analysis. Unstained gels will be transferred to PVDF membranes (EMD Millipore Corp., Billerica, MA USA) in electrode buffer containing 20% methanol and 0.1% SDS (Bolt and Mahoney, 1997), using a mini-transblot system (Bio-Rad) at a constant 80 mA overnight at 4°C (Kohn et al., 2007, 2011). The membranes will be dried and quickly stained in Ponceau S (Sigma-Aldrich, St. Louis, MO USA) to check for protein transfer, blocked with a synthetic blocker (CH-blok: Millipore) for 1 h, and then incubated with monoclonal antibodies (mAbs) overnight at 4ºC. Antibodies with known immunospecificity against MHC isoforms will be purchased

(as concentrates) from the Developmental Studies Hybridoma Bank (DSHB, University of Iowa). S58 is specific to the MHC-1 isoform (Miller et al., 1985) and was shown to reliably identify MHC-1 fibers in our previous study of adult *Monodelphis* (Rupert et al., 2014). Four of the mAbs are specific to rat MHC isoforms (Schiaffino et al., 1989): SC71 specific to MHC-2A, BF-35 specific to all MHC isoforms except MHC-2X, BF-F3 specific to MHC-2B, and B103 specific to MHC-neo. The immunospecificity of SC71 and BF-35 against both the MHC-2A and 2X isoforms was also confirmed in our previous study (Rupert et al., 2014), as was the specificity of the antibody 2F7 against the only MHC-2A isoform in adult *Monodelphis*. Working dilutions for each antibody (concentrates) will be 1:500 (0.2–0.5 μg/ml) depending on the original Ig concentration. Horse-radish peroxidase-linked rabbit (anti-mouse) will be used as a secondary antibody (1:10,000 dilution; Millipore) and following a 1–2 hr incubation, membranes will be washed twice in Tris Buffered Saline with Tween 20 (TBS-T) at 10 min each wash, reacted with chemiluminescence HRP substrate (5 min), and detected using the Fluor-Chem E Imaging System (Cell Biosciences, Santa Clara, CA USA) and additionally imaged on film (Kodak: Biomax Imaging Film).

rtPCR (qPCR)

Reverse transcriptase polymerase chain reaction (rtPCR) will be used to evaluate mRNA expression of the different MHC isoforms (Uber and Pette, 1993; Peuker and Pette, 1993; Peuker and Pette, 1997). The rtPCR technique (based on pairs of short oligonucleotide primers) allows for higher specificity than hybridization techniques (Pette et al., 1999) for expression identification of specific MHC isoforms. MHC bands (MHC-1, 2A, 2X, 2B, and MHC-neo) resolved on SDS-PAGE gels (see above) will be sectioned from the gels using a razor blade, placed in tubes containing a 5% acetic acid buffer, and sent to The Ohio State University for protein sequencing of the isoforms (sequencing of 1, 2A, and 2X is already complete). From the protein sequence data, sets of forward and reverse primers will be designed and made specific for each MHC isoform.

Small muscle samples of the FCL (100 mg) will be quickly immersed in conical tubes containing RNA Later Reagent (Ambion, Austin TX, USA) and stored at -20ºC. Total RNA (1.5 μg) will be extracted from the muscle samples in TRIZOL Reagent (GibcoBRL, Gaithersburg, MD, USA) and reverse transcribed with SuperScript II

protocols (Invitrogen, Life Technologies) using as primers a mixture of random hexamers (Toniolo et al., 2004, 2005, 2007) to synthesize the first-strand cDNAs. The cDNA produced will serve as templates for rtPCR reactions. Specifically, 200 ng of extracted cDNA will be amplified by rtPCR using one primer pair (forward and reverse). The rtPCR reactions will be standardized (e.g., cycle numbers and annealing temperature) for each MHC isoform and will be run for a total of ~3 min under the following optimized conditions: 27 cycles for 50 sec at 94°C, 12 cycles for 50 sec at annealing temperature (progressively reduced from 58ºC to 52°C), and the last series of cycles for 50 sec with an extension step at 72°C (Maccatrozzo et al., 2004; Toniolo et al., 2004). All reactions will be performed in 20 µl of a PCR solution: 1× PCR buffer (Gibco, BRL), 1.8 mmol/L $MgCl_2$, 0.1 mmol/L of each dNTPs, 0.5 µmol/L of each primer, 0.5 U Taq DNA polymerase and 1 µl cDNA (Toniolo et al., 2004). A yield of 5 µl from each PCR product then will be electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light (Maccatrozzo et al., 2004). To evaluate the quality of the extracted RNA and efficiency of the RT reaction, a fragment of β-actin cDNA from *Monodelphis* also will be amplified by a primer pair in each sample as an internal control (Toniolo et al., 2005). The β-actin will additionally serve as a loading standard to verify that the same amount of PCR product was loaded in each sample lane.

Tail Behavior Observations

Videographic data will be collected at the Texas Biomedical Research Institute (which houses the largest U.S. colony of captive *M. domestica*) to quantify use of their semi-prehensile tail. Over two separate four day periods, three digital video cameras (frame rate: 60 Hz) will be setup to record a total of juvenile (N=10) and adult (N=10) opossums overnight (1800–0900 h) to document movements involved in initiation of tail flexion and nest-building behaviors. Each camera will be placed ~0.70 m away from the cages, at a height of 1.10 m, and adjusted at an angle 33 deg to film four cages simultaneously. On the first night of filming for each animal, glass mason jars will be set in the cage to facilitate nest building in the jars. During the successive second, third, and fourth nights, the jars will be removed to observe more diverse actions of the tail. The frequencies of the following behaviors will be quantified to determine how often the young opossums flex/use their tail in comparison to the adults and how the movement behaviors differ: tail

coiling (flexing tail without handling/maneuvering material), lateral coiling (lateral flexing tail to move nest material or objects into a pile), and nest coiling (flexing tail around nesting material while building the nest) (Unger, 1982). The total percentage of time opossums perform these behaviors will be calculated and tested for statistical differences between juveniles and adults.

Supplemental Table 2: Morphological data for video recorded *M. domestica*.

Opossum	Sex	Age	Body mass (g)	Head-Body length (cm)	Tail length (cm)	Relative Tail Length
Juvenile						
M3mo	M	3 mo	48.6	12.2	7.5	0.61
M4mo	M	4 mo	58.9	14.0	7.4	0.53
M5mo	M	5 mo	114.2	15.0	7.0	0.47
M6mo	M	6 mo	102.0	16.0	5.0	0.31
F7mo	F	7 mo	64.8	13.5	8.2	0.61
			77.7±28.7	**14.1±1.4**	**7.0±1.2**	**0.51±0.12**
Adult						
F1yrA	F	1 yr	90.8	14.5	7.4	0.51
M1yrA	M	1 yr	141.0	18.8	10.1	0.54
F1yrB	F	1 yr	106.0	16.0	8.3	0.52
M1yrB	M	1 yr	144.0	19.5	10.1	0.52
F1yrC	F	1 yr	96.6	15.5	8.7	0.56
M2yr	M	2 yr	114.3	15.5	7.4	0.48
			115.5±22.5	**16.6±2.0**	**8.7±1.2**	**0.52±0.03**

Table 1. Custom designed forward and reverse primers for MHC in *M. domestica*.

Isoform	Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
MHC-1β	MYH7	CATCAAGGCCAAGGCTAATCTA	GTTGTGTCTCCTCTGCTTTACT
MHC-2A	MYH2	CACCCTGGAGGATCAAGTAAG	CCCTCTGAGTTGTCAGATCATT
MHC-2X	MYH1	GCCGCACTCTAGAAGATCAAG	CCTCTTAACAGCTGAGAAACTA
MHC-2B	MYH4	CCAAGGAGGAAGAGCAACAA	TGCCTCTTGATAGCTGAGAAAC
MHC-Neo	MYH8	AAAAGAAAGTCTTGCAAAGTCA	TTTATTCTTGATAAGTTGTTCA
MHC-Emb	MYH3	CACGGAGCAAGACAGAAGAA	TCCTAGAAAGCTGGGATACAATG

Table 2. Means (%) of mRNA in the tail regions of *M. domestica* across ontogeny.

		Proximal-mRNA %						Transitional-mRNA %						Distal-mRNA %					
Age	*n*	**1β**	**2A**	**2X**	**2B**	**Neo**	**Emb**	**1β**	**2A**	**2X**	**2B**	**Neo**	**Emb**	**1β**	**2A**	**2X**	**2B**	**Neo**	**Emb**
22 days*	3	**63.6**	**2.83**	**0.15**	**0.01**	**20.0**	**13.4**												
3 mo	3	75.9	5.03	2.37	14.2	2.19	0.37	72.5	9.34	1.31	11.5	4.35	0.94	47.5	11.6	3.52	27.2	8.48	1.68
5 mo	3	65.7	26.3	2.45	0.33	2.91	2.31	48.1	35.6	3.01	2.21	6.12	4.96	40.4	23.9	7.22	14.7	9.41	4.31
7 mo	3	70.9	0.95	3.69	21.7	2.32	0.48	53.7	35.9	1.24	1.32	5.57	2.29	45.4	34.7	2.35	4.91	9.38	3.28
13 mo	3	85.7	12.4	0.48	0.37	0.61	0.49	77.7	18.6	0.45	0.44	1.66	1.16	78.6	17.9	0.42	0.43	1.64	0.97
29 mo†	3	80.2	9.67	2.62	1.62	5.24	0.63												

n= number of qPCR runs
*Analyzed from a single homogenate stock made from the tail buds of 3 individuals
†mRNA was extracted from muscle tissue available for only the proximal tail region

Table 3. Means (%) of MHC isoform content in the tail regions of *M. domestica* across ontogeny.

		Proximal-MHC %					Transitional-MHC %					Distal-MHC %				
Age	N	**1β**	**2A**	**2X**	**2B**	**Neo**	**1β**	**2A**	**2X**	**2B**	**Neo**	**1β**	**2A**	**2X**	**2B**	**Neo**
22 days*	3	**11.8**	**49.4**	**10.6**	**23.6**	**4.6**										
3 mo	3	8.3 (3.8)	46.6 (13.0)	30.8 (11.6)	11.8 (0.2)	2.5 (2.1)	7.7 (0.2)	52.3 (7.2)	29.1 (6.6)	7.5 (1.9)	3.4 (2.8)	9.6 (3.4)	51.7 (8.1)	26.9 (7.7)	8.2 (6.2)	3.7 (4.4)
4 mo	3	8.9 (2.5)	52.2 (13.5)	27.7 (9.8)	8.3 (2.5)	2.9 (3.1)	9.0 (1.3)	55.8 (9.3)	25.6 (7.6)	7.3 (3.4)	2.4 (1.6)	8.0 (2.8)	55.1 (8.6)	22.3 (7.9)	11.4 (9.5)	3.2 (1.8)
5 mo	3	10.5 (3.1)	48.4 (21.3)	26.4 (14.7)	10.0 (9.4)	4.6 (0.9)	10.1 (1.9)	49.8 (13.6)	31.2 (6.8)	6.3 (2.7)	2.5 (2.2)	9.5 (4.3)	54.2 (7.4)	26.3 (3.3)	4.6 (2.4)	5.5 (5.7)
6 mo	3	11.1 (9.3)	48.0 (6.2)	26.3 (14.1)	11.2 (1.5)	3.3 (3.0)	10.6 (3.0)	53.1 (11.3)	23.6 (11.0)	10.9 (10.0)	1.8 (1.6)	10.5 (1.3)	45.1 (4.6)	28.5 (10.0)	10.5 (6.7)	5.4 (4.5)
7 mo	3	12.5 (2.9)	38.2 (12.9)	30.2 (16.7)	8.6 (3.3)	10.5 (4.3)	10.1 (0.8)	40.3 (4.7)	44.5 (3.3)	3.5 (5.0)	1.5 (2.1)	13.4 (3.5)	43.2 (0.4)	37.5 (12.3)	4.0 (5.7)	2.0 (2.8)
13 mo	3	13.4 (2.0)	44.1 (7.4)	38.2 (16.3)	0.9† (1.5)	3.4† (5.9)	11.6 (2.5)	45.9 (0.8)	37.3 (6.8)	1.7† (3.0)	3.4† (6.0)	15.6 (4.6)	50.7 (10.1)	30.1 (17.7)	0.0	3.6† (6.2)
29 mo	1	14.1	61.8	24.2	0.0	0.0	19.3	64.8	15.9	0.0	0.0	22.4	59.8	17.9	0.0	0.0

In parentheses is the standard deviation (s.d.)
N= number of individuals
*Analyzed from a single homogenate stock made from the tail buds of 3 individuals
†Found only in one male individual

Table 4. Percentages of nocturnal nesting activity for *M. domestica.*

Age Group	Sex	N	Nesting w/ tail (NT %)	Nesting w/o tail (N0 %)	Other activity (O %)*	Nesting Total (%)
3 mo	F/M	2	0.00	0.00	100	**0.00**
5 mo	M	2	0.16	0.42	99.4	**0.58**
7 mo	F	2	0.00	0.94	99.1	**0.94**
13 mo	F	2	11.1	1.55	87.4	**12.6**
29 mo	M	1	2.99	2.31	94.7	**5.30**

Values are calculated as whole percentages from 2–3 nights of independent observations
Values in bold are total nesting activity time percentage
*O: does not imply the animal was sleeping; includes grooming, scratching, tripodal stance, and repositioning

Table 5. Frequencies of tail-specific behaviors for *M. domestica.*

Age Group	Sex	N	Distal tail flexion (DF %)	Grasping/Carrying material (GC %)	Total observations (n)
3 mo	F/M	2	100 (52)	0.0 (0)	52
5 mo	M	2	96.2 (50)	3.81 (2)	52
7 mo	F	2	100 (9)	0.0 (0)	9
13 mo	F	2	33.3 (31)	66.7 (62)	93
29 mo	M	1	0.0 (0)	100 (36)	36

N=number of individuals; in parentheses are the number of independent observations
Values are calculated as whole percentages from 2–3 nights of independent observations

Figure 1. Photographs of the behavioral data collection environment showing camera placement (**A**), animal enclosures in the camera field-of-view (**B**), and graphical depictions of nest activity with the tail (NT) observed in five different age groups of *M. domestica* (**C**). Cameras were positioned to film (60 Hz) four animals simultaneously. Red photos indicate that filming was performed in infrared light conditions. Anatomical outlines of 3 mo, 5 mo, 7 mo, 13 mo, and 29 mo opossums illustrating both changes in body size (mass) and the total percentage of tail use for nest construction as reported in Table 4. Outlines (gray) are all illustrated as the right lateral view. Tail segments are shown in gradients of red to represent use of the tail for nest construction, where brighter shades of red indicate greater activity. Scale bar = 10 cm.

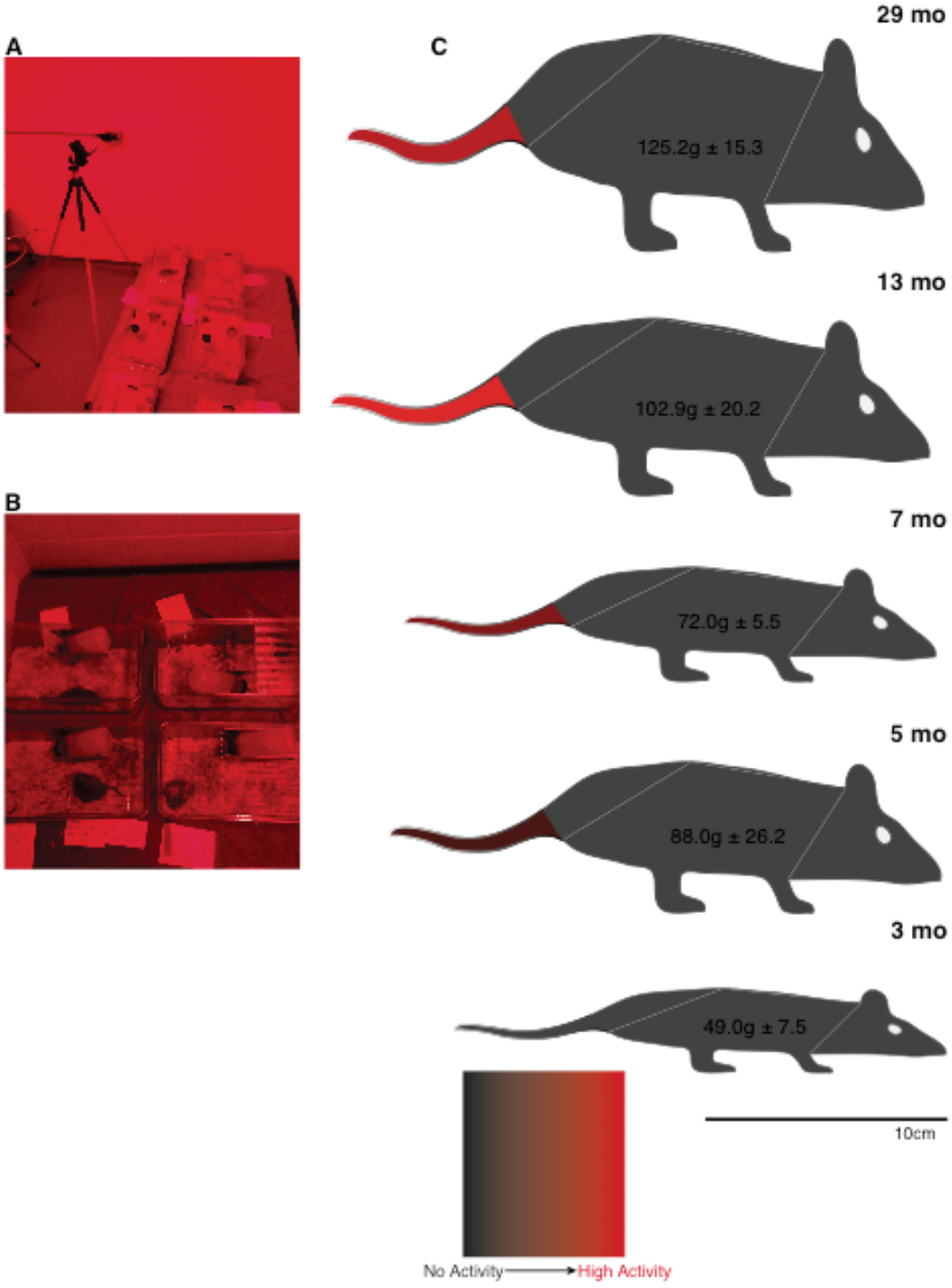
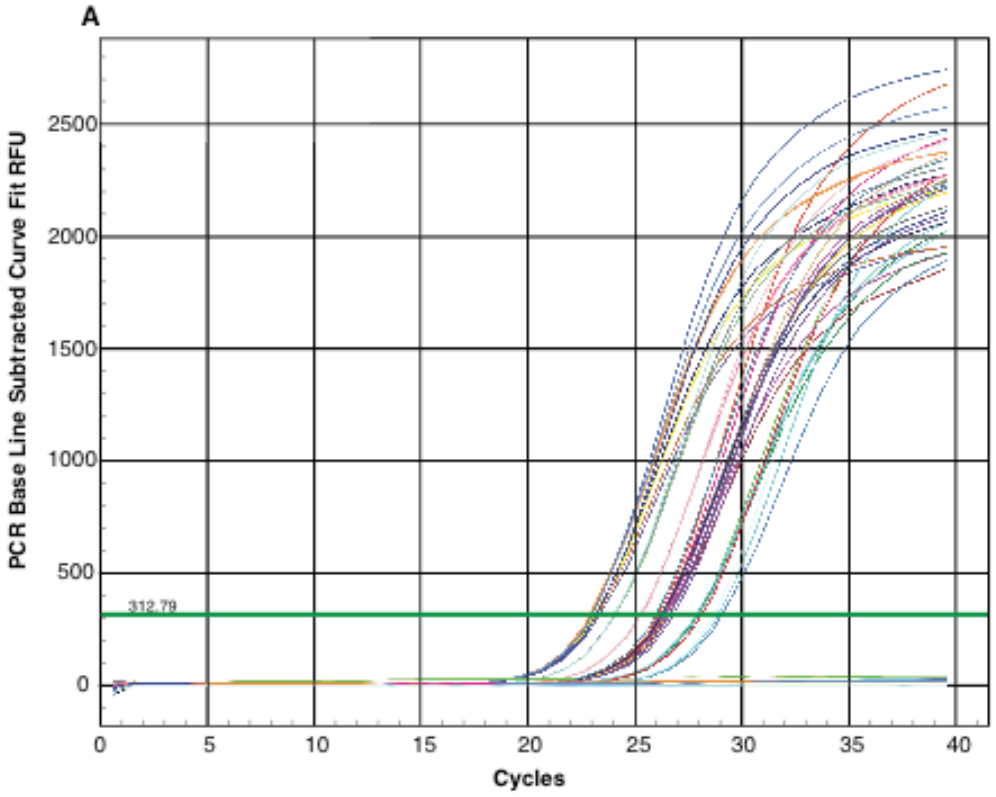

A
B
C
29 mo
125.2g ± 15.3
13 mo
102.9g ± 20.2
7 mo
72.0g ± 5.5
5 mo
88.0g ± 26.2
3 mo
49.0g ± 7.5
10cm
No Activity
High Activity

Figure 2. Example of qPCR cycle number for MHC isoforms (**A**), and a standard curve from which the quantity of mRNA was calibrated (**B**). Runs of qPCR were repeated 3x on extracted mRNA (cDNA) from one individual from the age groups: 22 days, 3 mo, 5 mo, 7 mo, 13 mo, and 29 mo. Standard curves were constructed from the mean data of each individual. The calibration equation derived from each linear regression was used to determine the total value of mRNA in each age group sample. Gene expression (relative) for each MHC isoform was then calculated as a percentage of the total mRNA quantified for each age group studied.


A
PCR Base Line Subtracted Curve Fit RFU
2500
2000
1500
1000
500
0
312.79
0
5
10
15
20
25
30
35
40
Cycles

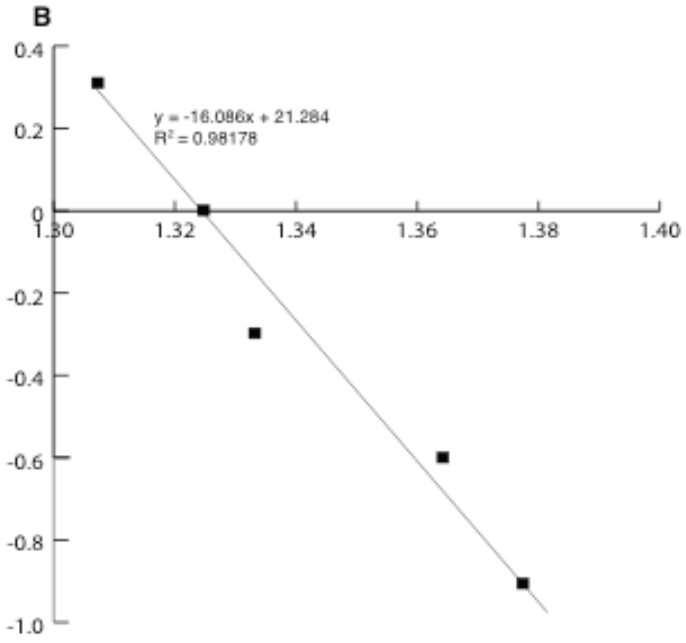

B
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0
y = -16.086x + 21.284
R² = 0.98178
1.30
1.32
1.34
1.36
1.38
1.40

Figure 3. Silver-stained gels identifying MHC isoforms in the *m. flexor caudae longus* (FCL) of juvenile and adult *M. domestica*. SDS-PAGE gel lanes are bracketed by age group with representative lanes from the tail regions shown (yellow text). The lane (left) containing MHC isoforms resolved for 1 day old opossums (from whole specimen homogenates) is shown as a band reference for the expression of developmental isoforms MHC-Emb and MHC-Neo. PX, proximal tail region; TS, transitional tail region; DS, distal tail region.

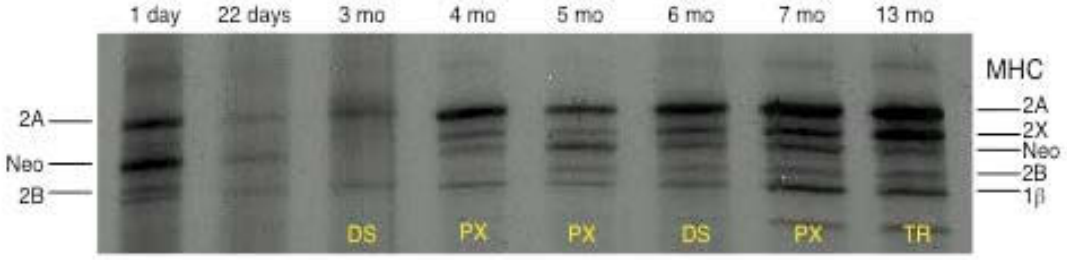

1 day
22 days
3 mo
4 mo
5 mo
6 mo
7 mo
13 mo
2A
Neo
2B
DS
PX
PX
DS
PX
TR
MHC
2A
2X
Neo
2B
1β

Figure 4. Cumulative percentage distribution of mRNA for each isoform (MHC gene expression) in the tail of *M. domestica* across ontogeny. See text for details of mRNA (%) quantification. Percentage mRNA for each MHC isoform and age group (except 29 mo: proximal region only) were determined and averaged across the proximal, transitional, and distal tail regions. Error bars are standard deviations (s.d.).

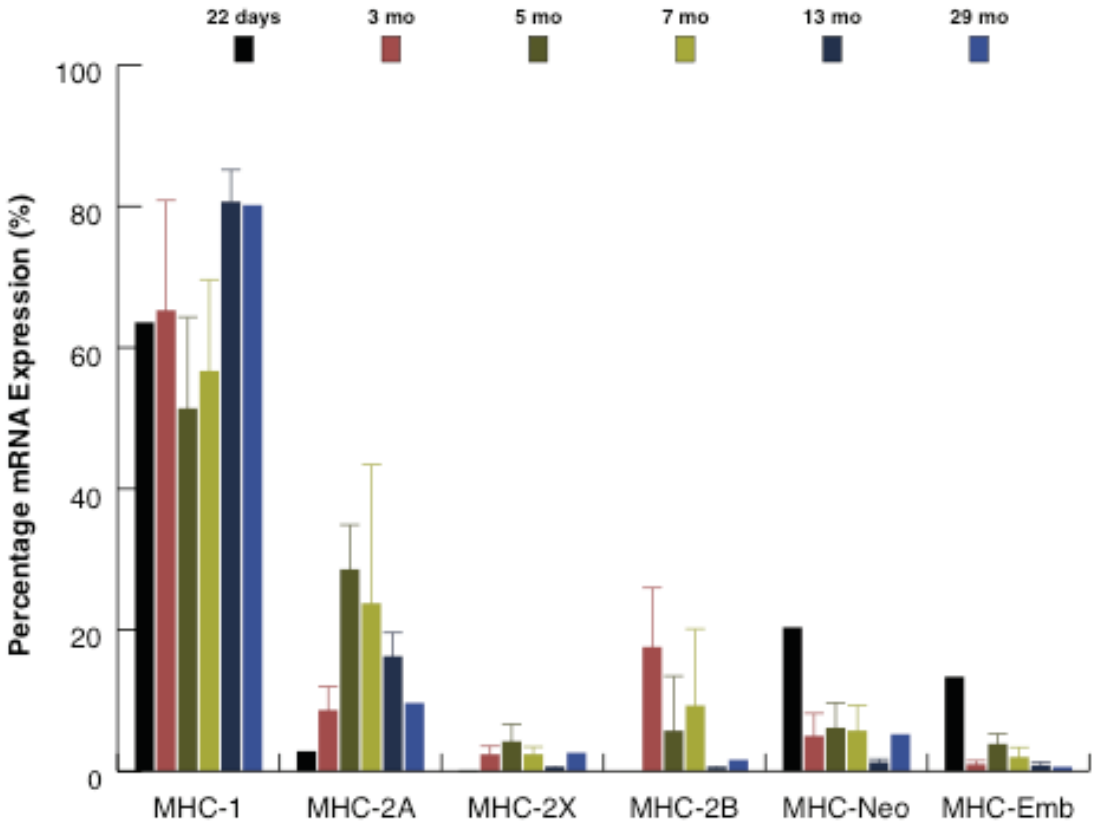

22 days
3 mo
5 mo
7 mo
13 mo
29 mo
Percentage mRNA Expression (%)
100
80
60
40
20
0
MHC-1
MHC-2A
MHC-2X
MHC-2B
MHC-Neo
MHC-Emb

Figure 5. Cumulative percentage distribution of MHC isoform content in the tail of *M. domestica* across ontogeny. Mean MHC isoform percentages from *N*=3 individuals per age group (except 29 mo) were determined by densitometric quantification and then averaged across the proximal, transitional, and distal tail regions. Error bars are standard deviations (s.d.). MHC-Emb is not represented because a functional protein isoform is not expressed in the tail.

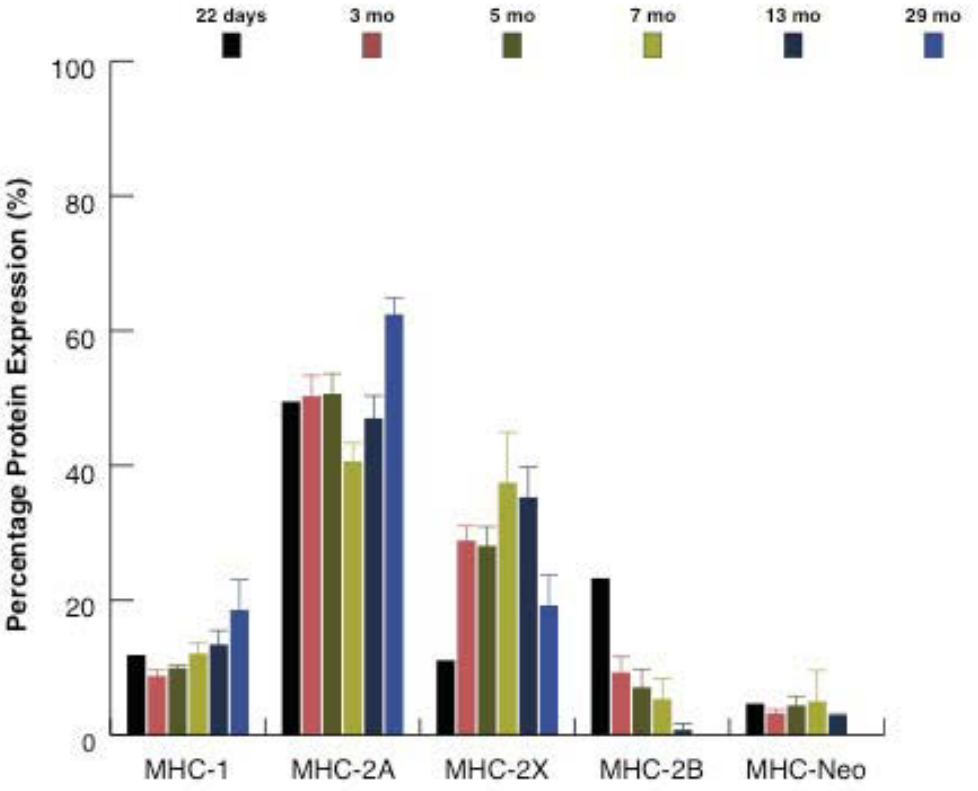

22 days
3 mo
5 mo
7 mo
13 mo
29 mo
Percentage Protein Expression (%)
100
80
60
40
20
0
MHC-1
MHC-2A
MHC-2X
MHC-2B
MHC-Neo

Figure 6. Proportional changes in expression of mRNA and MHC isoform content with ontogeny. Data for each age group shown are normalized to mRNA and MHC content quantified for each preceding age group. Data for the 3 month age group are normalized to those at 22 days old. Black bars are the change in mRNA. Gray bars are the change in MHC isoform content. *Percent change in mRNA for MHC-2B is 14x greater than shown due to its trace expression in the tail of 22 day old opossums. Axes have been scaled similarly for ease of comparison among isoforms.

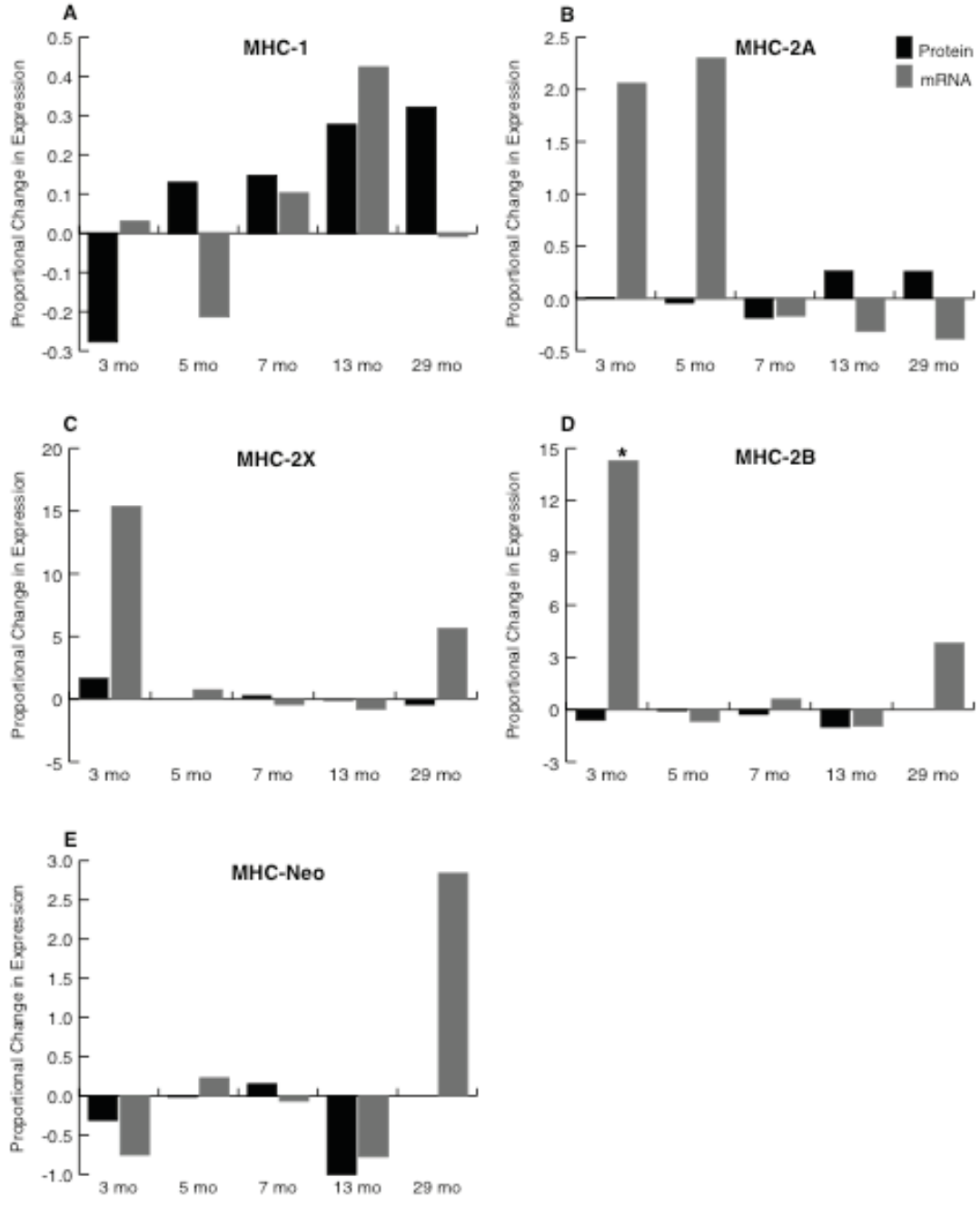

A
MHC-1
Proportional Change in Expression
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
3 mo
5 mo
7 mo
13 mo
29 mo
B
MHC-2A
Protein
mRNA
Proportional Change in Expression
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
3 mo
5 mo
7 mo
13 mo
29 mo
C
MHC-2X
Proportional Change in Expression
20
15
10
5
0
-5
3 mo
5 mo
7 mo
13 mo
29 mo
D
MHC-2B
*
Proportional Change in Expression
15
12
9
6
3
0
-3
3 mo
5 mo
7 mo
13 mo
29 mo
E
MHC-Neo
Proportional Change in Expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
3 mo
5 mo
7 mo
13 mo
29 mo

Figure 7. Relationship between MHC isoform content in the FCL and frequency of grasping/carrying (GC) of nesting materials by the tail across ontogeny. MHC isoform data are percentage expression from only the distal tail region. Scatter plots for each isoform are illustrated with a projected line of best fit by model I (LLS) regressions. Age groups are coded by colors defined in the legend. Panels are MHC-1β (**A**), MHC-2A (**B**), MHC-2X (**C**), and MHC-2B (**D**), and MHC-Neo (**E**).

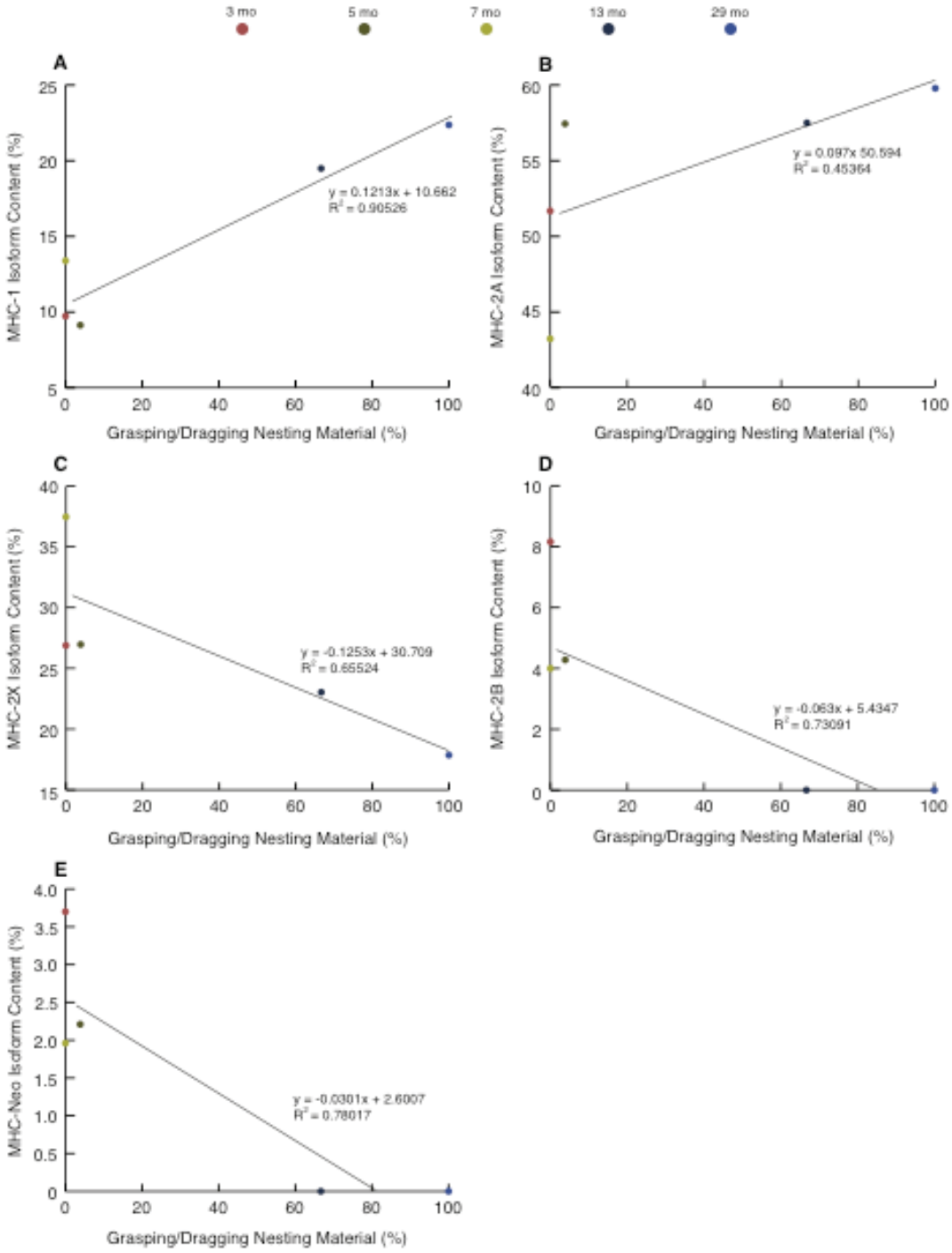
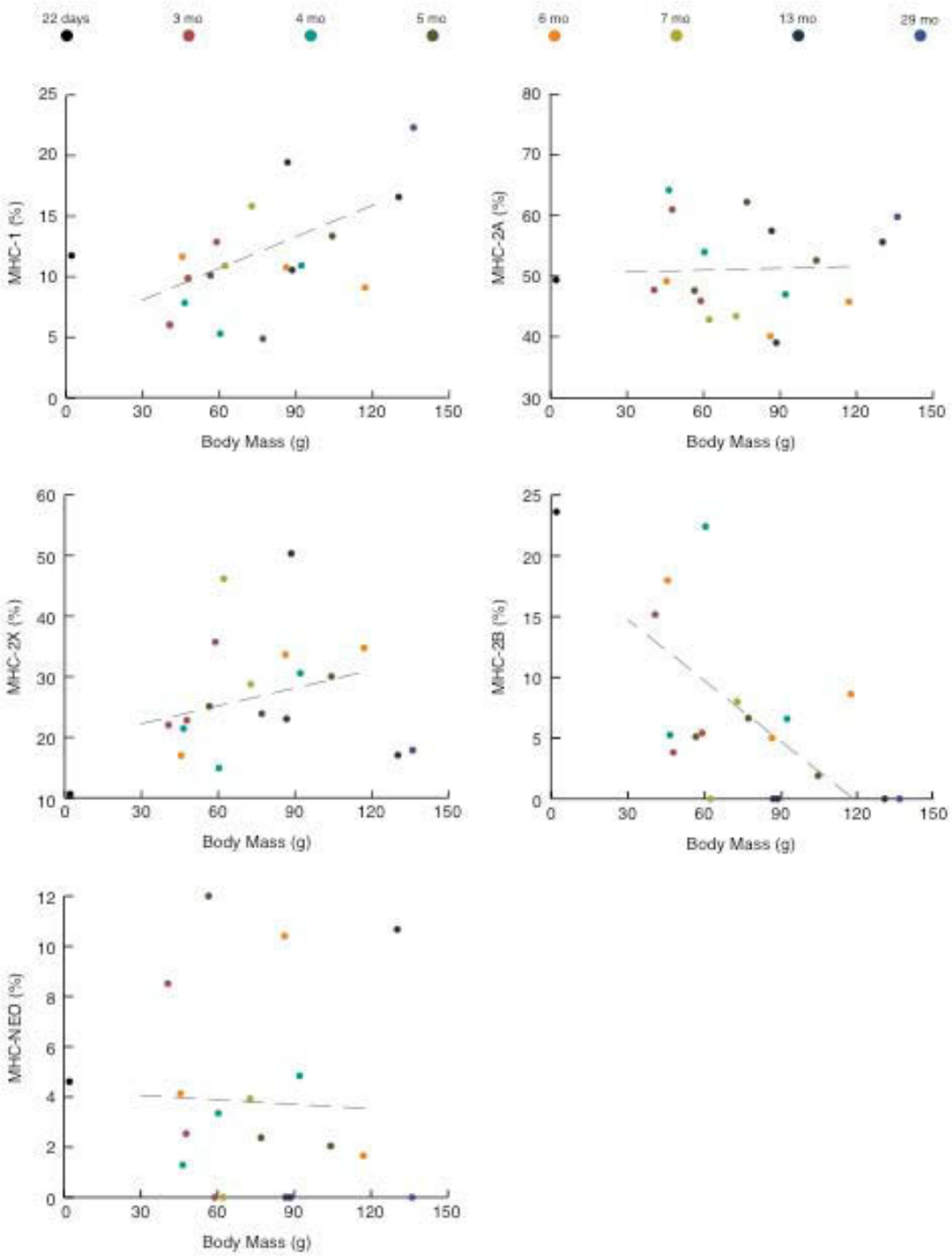


Figure 8. Relationship between MHC isoform content in the FCL and body mass across

ontogeny. MHC isoform data are percentage expression from only the distal tail region. Scatter plots for each isoform are illustrated with a projected line of best fit by model I (LLS) regressions. Age groups are color-coded to match those used in Figure 7. Panels are MHC-1β (Pearson's r_{17} −0.497, p=0.03) (**A**), MHC-2A (Pearson's r_{17} −0.048, p=0.84) (**B**), MHC-2X (Pearson's r_{17} −0.236, p=0.33) (**C**), and MHC-2B (Pearson's r_{17} +0.639, p<0.01) (**D**), and MHC-Neo (Pearson's r_{17} +0.069, p=0.78) (**E**).


22 days
3 mo
4 mo
5 mo
6 mo
7 mo
13 mo
29 mo
MHC-1 (%)
MHC-2A (%)
MHC-2X (%)
MHC-2B (%)
MHC-NEO (%)
Body Mass (g)