Breaking Constraint: Axial Patterning in *Trichechus* (Mammalia: Sirenia)

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ABSTRACT

Meristic variation is often limited in serially homologous systems with high internal differentiation and high developmental modularity. The mammalian neck, an extreme example, has a fixed (at seven) count of diversely specialized segments. Imposition of the mammalian cervical constraint has been tentatively linked to the origin of the diaphragm, which is muscularized by cells that migrate from cervical somites during development. With six cervical vertebrae, the genus *Trichechus* (manatee) has apparently broken this constraint, although the mechanism of constraint escape is unknown. Hypotheses for the developmental origin of *Trichechus* cervical morphology include cervical rib 7 repatterning, a primaxial/abaxial patterning shift, and local homeosis at the cervical/thoracic boundary. We tested predictions of these hypotheses by documenting vertebral morphology, axial ossification patterns, regionalization of the postcranial skeleton, and the relationship of thoracic ribs to sternal subunits in a large data set of fetal and adult *Trichechus* and *Dugong* specimens. These observations forced rejection of all three hypotheses. We propose alternatively that a global slowing of the rate of somitogenesis reduced somite count and disrupted alignment of *Hox*-generated anatomical markers relative to somite (and vertebral) boundaries throughout the *Trichechus* column. This hypothesis is consistent with observations of the full range of traditional cervical morphologies in the six cervical vertebrae, conserved postcranial proportions, and column-wide reduction in count relative to its sister taxon, *Dugong*. It also suggests that the origin of the mammalian cervical constraint lies in patterning, not in count, and that *Trichechus* and the tree sloths have broken the constraint using different developmental mechanisms.
INTRODUCTION

Serially homologous systems with high internal differentiation often show limited variation in element count (Darwin 1859, Bateson 1894, Hughes 2007). This pattern is thought to be the product of the modular organization of the developmental process (e.g. Beldade et al. 2002; Brakefield 2006, 2011; Marroig et al. 2009). Ancestrally, all elements of serially homologous systems typically share the same morphology, and by inference the same developmental history. They can be considered to belong to the same module. In descendant taxa, downstream developmental steps may be added in subunits of the system, allowing regional specializations. If adaptive, these novel submodules may be resistant to incorporation or expansion into adjacent, differently specialized submodules, with resulting meristic constraint.

The axial skeleton is a classic example of a serially homologous system. Its component vertebrae have their developmental origin in somites, which are generated sequentially from the presomitic paraxial mesoderm during somitogenesis. Total vertebral counts vary widely among vertebrates (Richardson et al. 1998, Ward and Brainerd 2007, Müller et al. 2010), indicating that axis elongation and segmentation rate, processes that govern somite production, must also be variable (Gomez and Pourquié 2008, Bénazéraf and Pourquié 2013). Tetrapods have differentiated cervical, dorsal, sacral, and caudal vertebral subunits, or series. The distinctive morphology of each series is the product of the differential regional expression of Hox and other patterning genes, identifying vertebral series as developmental modules (e.g. Burke et al. 1995, Wellik 2007, 2009). In most taxa, boundaries between adjacent series are moveable, and counts are variable (Müller et al. 2010). These variations may have diverse and/or multiple origins arising from changes in axis length, segmentation rate, and patterning boundaries. The interactions among these
processes are subjects of active research (e.g. Schröter and Oates 2010, Harima et al. 2013, Bénazéraf and Pourquié 2013).

The mammalian cervical series is unusual in that its count has been effectively fixed at seven since the Triassic (Jenkins 1971). It is also unusual in its high internal differentiation, evidenced by the high morphological variation among its vertebrae, the divergent destinations of the migratory muscle precursor cells that originate in its somites (Birchmeier and Brohmann 2000), and the presence of multiple, diverse suites of Hox patterning genes (Kessel 1992). This high internal differentiation has been hypothesized to restrict homeotic movement of the cervical/thoracic boundary (Buchholtz 2014), as the repatterning of cervical somites could compromise important developmental pathways. Among these pathways is the migration of the mid-cervical somitic premuscle cells that muscularize the diaphragm, a structure with significant functional roles in respiration and locomotion (Hirasawa and Kuratani 2013, Buchholtz 2014). Three mammalian genera with unusual anatomical features, lifestyles, and low metabolic rates have apparently escaped the cervical constraint: the tree sloths Bradypus (8-10 cervical vertebrae) and Choloepus (5-6), and the manatee Trichechus (6). The axial development and morphology of these genera offer opportunities to examine how evolutionarily imposed barriers to adaptive morphologies may be circumvented.

Sternal and costal morphology is critical to the examination of cervical patterning because the most anterior rib with a sternal articulation defines cervical/thoracic boundary. The key first thoracic rib (TR1) is unique in both morphology and development. TR1 is the only rib that articulates exclusively with the sternal manubrium, which in eutherians is likely a composite structure formed from the evolutionary integration of the reduced interclavicle, the ancestral mammalian presternum (confusingly sometimes also called the manubrium),
and the anterior terminations of the paired sternal bands (Parker 1868, Luo et al. 2007, Rodriguez-Vasquez et al. 2013). The developmental linkage of TR1 with the manubrium is suggested by the loss of both TR1 and the manubrium in Hox5 paralogous mutant mice (McIntyre et al. 2007). TR1 is also the only abaxial rib, as indicated by the presence of an abaxial label in the Prx1Cre transgenic mouse (Durland et al. 2008). TR2 articulates with the sternum just posterior to the composite manubrium, near or coincident with the anterior border of the sternal bands. The locations of TR1 and TR2 sternal articulations therefore provide morphological landmarks that can be useful in evaluating rib homology.

Non-traditional cervical counts in Bradypus and Choloepus are accompanied by extreme variability of vertebral, sternal, costal, and sacral anatomy. A hypothesis for the origin of this anatomy by homeotic movement of the cervical / thoracic boundary (Galis 1999, Galis et al. 2006, Varela-Lasheras et al. 2011) appears to be inconsistent with the disruption of these multiple systems. An alternative hypothesis (Buchholtz and Stepien 2009, Hautier et al. 2010, Buchholtz 2014) proposes anteroposterior repatterning of abaxial, but not primaxial, tissues. This interpretation implies that adaptive morphologies (elongated or foreshortened necks) have been achieved despite retention of the traditional primaxial cervical/thoracic boundary, but at the cost of morphological disruptions at the lateral somitic frontier (Burke and Nowicki 2003) where primaxial and abaxial tissues interact.

In contrast to tree sloths, Trichechus displays almost no variability in its cervical count and limited variability in its costal and sternal anatomy. Here we examine alternative hypotheses for the developmental route that allowed the evolutionary reduction of Trichechus cervical count, using its traditionally patterned sister genus, Dugong, as the primary comparison taxon. An early hypothesis (Murie 1872,1880; Chapman 1875; Harris 1939) proposed the deletion ("excalation") of a Trichechus cervical vertebra after
segmentation, with the result that two spinal nerves exit between adjacent vertebrae. This proposal is inconsistent with current understanding of segmentation (e.g. Bénazéraf and Pourquié 2013) and with manatee neural anatomy (Giffin and Gillett 1996). Three other hypotheses, each making different predictions, were tested against observations of fetal and adult vertebral, costal, and sternal anatomy and with previously published descriptions of brachial plexus anatomy.

1. Cervical rib (CR)7 repatterning: the small, fused cervical rib component of vertebra (V)7 has been repatterned as a full (thoracic) rib that articulates with the sternum. The occurrence of CR7 ribs with variable penetration in Hoxa4 mutant mice (Horan et al. 1994) and in up to 3% of some human populations (Berwin et al. 2009) suggest the existence of a CR7 developmental pathway independent of other cervical ribs. Predictions: thoracic ribs associated with V8 and V9 will retain their traditional articulation sites with and posterior to the manubrium, but the CR7 rib will articulate at a novel, more anterior manubrial location. The brachial plexus will retain its traditional location relative to the V7/V8 transition.

2. Primaxial/abaxial repatterning: abaxial but not primaxial components of the axial skeleton have been repatterned. An abaxial patterning shift has been hypothesized as the origin of disjunct anatomy at vertebral series boundaries and non-traditional cervical counts in tree sloths (Buchholtz and Stepien 2009). Predictions: the primaxial V7 vertebra and proximal rib will have cervical anatomy and ossification sequence, but the abaxial distal V7 (and more posterior) ribs will be repatterned to reflect anatomy typical of the next most posterior segment. Morphology may be disrupted at the proximal /distal rib boundaries. Because both the sternum and the limbs are also
abaxial, they, and the brachial plexus, will be anteriorly displaced relative to the V7/V8 vertebral transition.

3. Local homeosis at the cervical/thoracic boundary: both primaxial and abaxial components of cervical segment 7 have been repatterned to thoracic anatomy. Homeosis resulting from alteration of Hox expression domains is associated with functional and phylogenetic transformation of the column (Burke et al. 1995), and can also be induced experimentally (e.g. Krumlauf 1994, Li and Shiota 1999). Predictions: V7 will have thoracic morphology and ossification pattern, and bear a full rib that articulates at the traditional TR1/manubrium articulation site. More anterior cervical vertebrae and all thoracic vertebrae will be unaffected, although thoracic count will be increased by one. The sternum and brachial plexus will be anteriorly displaced relative to the traditional V7/V8 vertebral transition.

Because our observations of manatee anatomy are inconsistent with all three of these hypotheses, we also propose a fourth, working hypothesis:

4) A reduction in the rate of somitogenesis has disrupted the alignment of segment boundaries and patterning domains throughout the column. Predictions: relationships among morphological transitions (vertebral, costal, sternal, neural) will be retained, but will be globally displaced relative to somite boundaries (Schröter and Oates 2010). In manatees, where vertebral count is reduced relative to outgroups, anatomical transitions of cervical vertebrae typically distributed among seven vertebrae will be distributed instead among six. The cervical/thoracic transition will occur between V6 and V7, and will retain its typical relationship to the limb, the brachial plexus, and the sternum.
MATERIALS AND METHODS

Skeletons of *Trichechus manatus latirostris*, *T. senegalensis*, *Dugong dugon*, and *Dusisiren jordani* were examined at natural history museums (Table 1). Fetal *T. manatus latirostris* specimens in the collections of the Marine Mammal Pathobiology Laboratory (St. Petersburg, FL) were scanned at the University of Florida College of Veterinary Medicine in Gainesville at 0.5 mm width and visualized using the software programs AMIRA (FEI Visualization Software Group) and Osirix. Michael Walsh and Alex Costidis of the University of Florida College of Veterinary Medicine provided an additional dataset of a neonate *T. manatus latirostris* scanned at 3 mm. The single *D. dugon* fetus, scanned at 2 mm, is from the research collection of Darlene Ketten of the Woods Hole Oceanographic Institution. Total body lengths (TL) of the fetuses were measured from the scans.


Vertebrae were counted, allocated to series, and photographed from anterior aspect. Centrum lengths (CL) were measured ventrally. Cervical (C) vertebrae lie anterior to the first vertebra bearing a full rib that articulates with the sternum. Thoracic (T) vertebrae bear articulating ribs, but because they intergrade gradationally with lumbars (L) and sacrals (S) when present, they were grouped as T/L in manatees and T/L/S in dugongs. Ventral facets
for hemal arches identify the first caudal (Cd) vertebra. The locations of the cervical/thoracic boundary and the precaudal/caudal boundaries were calculated as percent of total centrum length (TCL, the sum of all centra in the column) in the rare complete skeletons (Trichechus N = 7; Dugong N = 2) and in the nearly complete skeletons (Trichechus N = 23; Dugong N = 3) evaluated as lacking no more than two terminal caudals. For nearly complete specimens, estimates of the CL of missing vertebrae were included in TCL. Estimated missing CL never exceeded 1.1% of TCL.

Photographs of the anterior surfaces of cervical and the first thoracic vertebrae were used to evaluate morphological variation across the cervical series (Trichechus N = 86; Dugong N =10). Geometric morphometric analysis was used to quantify vertebral shape of the cervical and first thoracic vertebrae of a subset of six Trichechus manatus and six Dugong dugon specimens. The software package tpsDIG2 (Rohlf 2013) was used to digitize fixed landmarks to locate discrete morphological features and sliding semilandmarks to capture overall shape. The divergent morphology of the atlas and axis dictated that a two-step analysis be used. A preliminary analysis of C1-T1 used seven fixed and 10 semilandmarks followed by a second analysis of C3-T1 that used 10 fixed and 30 semilandmarks. The software package MorphoJ (Klingenberg 2011) was used to carry out Procrustes fit and principal components analysis.

Vertebral ossification centers of the neonate and fetal manatees (N=11) were isolated with the software AMIRA. Threshold differences between soft and ossified tissues varied somewhat among specimens, possibly reflecting different preservation histories, but were always substantial. The distribution of ossification centers was then used to reconstruct column ossification sequence with change in total body length. Images of fetal sternae
reconstructed from the fetal CT scans and the sternae of postnatal individuals of different total body length were used to reconstruct sternal ontogeny.

RESULTS

Vertebral count and length. Each *Trichechus manatus* skeleton in this database (N=86) has six cervical vertebrae, and each *Dugong dugon* specimen (N=10) has seven cervical vertebrae using the classical definition of TR1/sternal articulation to identify the first thoracic vertebra (Table II). Each skeleton of the comparative manatee species *T. senegalensis* also has six cervicals, and the single fossil dugongid *Duisiren jordani* has seven. T/L (*Trichechus*), T/L/S (*Dugong*), and caudal counts are variable within small ranges (Table II). Total vertebral counts in *Trichechus* average 85.9% those of *Dugong*, and mean series counts in *Trichechus* are 85.7% (cervical), 83.9% (thoracic/lumbar or thoracic/lumbar/sacral), and 88.6% (caudal) those of *Dugong*. The fractions of TCL at the cervical/thoracic and precaudal/caudal boundaries of both genera were remarkably stable in complete, nearly complete, and combined (complete + nearly complete) data sets, and were statistically indistinguishable between and within genera (t tests, all probabilities > 0.05, Table II).

Vertebral morphology. The atlas and axis of *Trichechus* are robust, reflecting their functional roles in skull articulation (Fig. 1). The mid cervicals (V3-V5) are gracile, with very short centrum lengths and boxy outlines. Neural arches are thin and often incomplete dorsally in juveniles; neural spines are absent to very short. Transverse processes extend distally from their origins at the ventral centrum base, increasing in width, in differentiation of the process base from the centrum, and in ventral inclination from V3 to V5. Transverse foramina are almost universally present in V3-V5, although their lateral boundaries may be incomplete, especially in juveniles. In rare individuals (Fig. 2A), V5 transverse process
shape mimics that typical of V6. V6 is more robust than V3-V5, and centrum length is about 1.5 times as long. Additionally, transverse processes are longer and originate more dorsally, from the base of the neural arch as well as from the centrum. V6 transverse foramina are variably reduced or absent, with loss or fusion of the ventral bar. Fusion lines of this bar, a homolog of the cervical rib capitulum (Cave 1975), are sometimes visible (Fig. 2B). Hemifacets are present at the ventrolateral borders of the V6 posterior centrum faces for the TR1 capitula (Fig. 2B,C), and very rarely on the transverse processes for the TR1 tubacula (Fig. 2C). V7(=T1) is easily distinguished from the cervicals by the presence of a robust neural spine and by the origin of its transverse processes from the neural arches alone. There are articular facets for the TR1 tubacula on the lateral extremities of the transverse processes, and hemifacets at the ventral border of the centrum for TR1 capitula anteriorly and TR2 capitula posteriorly (Fig. 2D). Among the 86 Trichechus cervical series examined, two showed V2/V3 fusion, one showed V1/V2/V3 fusion, and one showed V4/V5 fusion. There was no statistical difference in left/right symmetry among cervical vertebrae in the subset analyzed (N=10, data not shown).

The Dugong atlas and axis largely mimic those of Trichechus. The remaining five cervical vertebrae show transitions in morphology that are parallel to, but somewhat differently distributed, than those of manatees. Dugong V3-V5 are small, with short centrum lengths and boxy shapes, but the transverse process transitions in increased width, differentiation of the process base, and ventral inclination occur later in the series than in Trichechus, and extend to V6 as well. The more dorsal location of the base of the transverse process occurs first at V7, not V6 as in the manatee. Similarly, the reduction or fusion of the ventral bar of the transverse process, the reduction of the transverse foramen, and the first occurrence of hemifacets for the TR1 capitula all occur on V7, with the result that it resembles the manatee V6 in shape. The dugong V8(=T1) mimics the manatee V7(=T1) in the presence of
a definitive neural spine and articulation facets for the TR1 tubercula; it is the first vertebra to show centrum elongation.

The first three axes (PCs) of the preliminary principal components analysis of the combined C1-T1 *Trichechus* and *Dugong* data set explained 57.6%, 18.3%, and 10.4% of the variation in the sample, respectively; all other axes explained less than 6% (Fig. 3A). Clusters for the atlas and the axis are separated from a third cluster of all remaining vertebrae on PC1, and the atlas is separated from the axis by PC2. Within each cluster, manatees are separated from dugongs on PC2. The first axis (PC1) reflects primarily location of the anterior zygapophysis, length of the neural spine, and dorsoventral position of the transverse process. The second axis (PC2) reflects primarily length of the transverse process, inclination of the anterior zygapophysis, and centrum height.

The first three principal components of the separate analysis of C3-T1 data set explained 45.5%, 18.1%, and 11.3% of sample variation; additional axes explained less than 4% (Fig. 3B). Vertebrae are distributed in axial order along PC1, reflecting primarily changes in the breadth of the transverse process, location of the anterior zygapophysis, and the shape of the centrum. Although the shapes of the most anterior cervicals (V3, V4) of manatees and dugongs are quite similar, the mid cervicals (V4, V5) of manatees are distinctly more “thoracic” in shape than those of dugongs. Shapes of the manatee V6 and V7 closely resemble dugong vertebrae one full axial position more posterior. Again, dugongs are separated from manatees on PC2, which reflects primarily inclination of the anterior zygapophysis and centrum height.

**Vertebral ossification sequence in *Trichechus***. In fetal *Trichechus* specimens, the onset of neural arch ossification precedes centrum ossification, and is bimodal in pattern. The
8.4cm fetus (Fig. 4A) has cartilaginous condensations of the atlas and mid thoracic neural arches, but lacks any ossification. The 12.9cm fetus has ossified atlas, axis, and mid thoracic neural arches (Fig. 4B). With increasing size, ossification of neural arches moves posteriorly from the axis and both anteriorly and posteriorly from the thorax. Anteriorly, the midcervical arches are the last to ossify (Fig. 4C). Because neural arch ossification precedes centrum ossification in the neck, the necks of small fetuses have ossified neural arches only. Posteriorly, neural arch ossification progresses very slowly past the anterior tail, lagging behind centrum ossification.

The ossification of centra starts in the midcolumn before neural arch ossification is complete. Multiple midcolumn centra appear to ossify rapidly: none are ossified in the 8.4cm fetus, but 29 (V8-V36=T2-Cd17) are ossified in the 12.9cm fetus (Fig. 4B). Additional centra are ossified at the anterior and posterior ends of this anterior thoracic – mid caudal unit in progressively larger specimens. There is no deviation from this pattern across the cervical/thoracic boundary: the centra of V8 (Fig. 4B), V7 (Fig. 4C) and then V6 (Fig. 4D) ossify in posterior to anterior sequence. Subsequently, the minute V3, V4 and V5 centra appear in variable sequence in different specimens (Fig. 4E, F). In contrast to the pattern reported in other mammals (Hautier et al. 2010, in press), centra of the atlas and axis ossify after those of more posterior cervicals (Fig. 4F). Ossification of the axial skeleton of the single dugong fetus (est. TL=72cm) is essentially complete.

**Sternal structure and ontogeny.** The adult *Trichechus* sternum lacks overt subdivision into the manubrium, metasternum and xiphisternum typical of most mammals, but reconstruction of sternal ontogeny from fetal scans reveals its composite structure. Fetuses less than 15cm in TL lack sternal ossifications. The first sternal ossifications are bilateral and circular (Fig. 5A), subsequently fusing across the midline (Fig. 5B), and then later forming the lateral
components of the adult sternum. Additional anterior and posterior midline ossifications join this fused central unit in all fetal individuals over 30cm in body length (Fig. 5C). Areas of low density are visible at the margins of the sternum and between sternal subunits in the 33cm specimen when viewed with maximum intensity projection (Fig. 5D). Subunit boundaries are obliterated in all larger fetuses and in all postnatal individuals (Fig. 5E-H). Postnatal sternae have shallow articulation facets for two (rarely three) cartilaginous sternal ribs at locations corresponding to the anterior and posteriormost margins of the fused central unit (Fig. 5H).

Fused bilateral ossifications also form the central unit of the sternum of the single Dugong fetal specimen (est. TL=72cm, Fig. 5I). A posterior midline ossification is present but not yet incorporated into the sternum. A transverse suture at the level of this gap was figured in a juvenile Dugong (Flower 1885, Fig. 42) and is identifiable in some adult Dugong sternae (e.g. Fig. 5J). It corresponds in location to the manubrium/metasternum junction (angulus sterni) identified by Kaiser (1974, Plate 55). Postnatal Dugong sternae are narrower than those of Trichechus, and articulate with three (rarely four) cartilaginous sternal ribs (Home 1820, Freund 1905, Domning 1977; Fig. 5J). The second rib articulates at or just anterior to the manubrium/metasternum junction (Fig. 5J).

**Brachial plexus location and morphology.** The brachial plexus of Trichechus manatus has been described at least four times (Murie 1872, 1880; Chapman 1875, Harris 1939, Giffin and Gillett 1996) with different degrees of detail. All reports agree on the general form and location of the plexus, which possesses four major roots. The most posterior of these major roots exits from the vertebral column between V6 and V7(=T1). A fifth, smaller root originates behind the first thoracic vertebra. This plexus is essentially identical to that of a “generic” mammal in the number of roots and the distribution of trunks, divisions and peripheral nerves, and in location with respect to the cervical/thoracic boundary as defined
by the TR1/sternal articulation. It differs primarily in that this location occurs one segment anterior to that seen in most mammals. On the basis of this anterior location, Murie (1872, 1880) Chapman (1875), and Harris (1939) inferred the ontogenetic deletion of vertebra 3, and interpreted the spinal nerve(s) originating between V2 and V3 as two separate nerves. As a result, they assigned the more posterior nerves a higher segment number than did Giffin and Gillett (1996).

**DISCUSSION**

**Testing competing hypotheses.** We test three alternative hypotheses for the developmental origin of *Trichechus* cervical anatomy using observations of its vertebral, sternal, and costal anatomy and comparisons to the anatomy of its living sister genus *Dugong*. These observations dictate rejection of each of the three hypotheses.

Hypothesis 1. Cervical rib 7 repatterning suggests that the typically reduced and fused eutherian CR7 rib has been remodeled as an elongate and moveable (thoracic) rib with a sternal articulation. Because sternal repatterning is not invoked, the additional rib is predicted to articulate with the sternum anterior to the classical V8=T1 rib articulation site, which has a unique and conserved relationship to the sternal manubrium. No movement of the limb or brachial plexus is predicted.

Reconstruction of the manatee sternal ontogeny indicates that the sternum is composed of four subunits: bilateral circular ossifications that join in the midline, and additional large anterior and elongate posterior subunits. The location and positional relationships of the central and posterior subunits correspond closely to those of the paired mesenchymal condensations of the manubrium and the sternal bands described in developmental and
comparative studies (Parker 1868, Paterson 1904, Gladstone and Wakeley 1932b, Klima 1987, Rodríguez-Vázquez et al. 2013) of living mammals. The articulations of TR1 at the axial level of the bilateral ossifications, and of TR2 at their posterior boundary in both *Trichechus* and *Dugong* also support this identification. Further, the similarity of this pattern in the two genera argues against a repatterning in *Trichechus* alone. The identity of the large anterior sternal subunit is less definitive, but its shape and position are similar to that of the interclavicle in ancestral mammals (Luo et al. 2007, Sereno 2006). This identification is also consistent with the articulation of the clavicles (when present) at the anterior manubrial border of eutherians. Despite the large size of the anterior sternal subunit in *Trichechus*, not a single specimen in this large data set exhibits a rib articulation anterior to the axial level of the central ossifications, contra to hypothesis predictions. The possibility of such an articulation is supported by the observation of three manubrial ribs in rare human individuals with fully developed CR7 ribs (Gladstone and Wakeley 1932a, p. 353).

The CR7 repatterning hypothesis also predicts alteration of the cervical/thoracic border, but not alteration of the location of the limb or brachial plexus relative to vertebral count. The observed anterior displacement of the plexus is a further argument against this hypothesis for the developmental origin of *Trichechus* cervical anatomy.

Hypothesis 2. Primaxial/abaxial repatterning predicts that the patterning of abaxial tissues (limbs, distal ribs, sternum) has been shifted anterior by one segment relative to that of the primaxial tissues (vertebrae, proximal ribs), which retain traditional patterning. Anterior displacement of the limbs is clear, based on the anteriorly shifted location of the brachial plexus, which is recruited from spinal cord neurons by the abaxial limb bud. However, the predicted skeletal disruptions at the primaxial/abaxial boundary of rib subunits, common in tree sloths, are not observed. Similarly, the *Trichechus* sternum lacks apparent disruption,
also frequently observed in tree sloths, and its rib articulations closely mirror those of the traditionally patterned *Dugong*. Unfortunately, the lack of a sacrum makes observation of primaxial/abaxial relationships at the level of the pelvic girdle impossible.

Primaxial/abaxial repatterning also predicts that the V7 centrum retains cervical ossification sequence, despite its articulation with a full (thoracic) rib. Centrum ossification sequence is highly conserved in mammals, and is delayed in the cervical series relative to the thoracic series. Hautier et al. (2010, and in press) proposed the use of this delay as an indicator of the cervical/thoracic developmental boundary. In *Trichechus*, however, the anteriormost thoracic centrum (V7) ossifies in sequence with those more posterior to it, and not with the small anterior cervical centra. Like observations of sternal anatomy, the pattern of axial ossification fails to support the primaxial/abaxial repatterning hypothesis.

The observed delay in the ossification of the *Trichechus* atlas and axis centra is highly unusual with respect to other mammals (Hautier et al. 2010, and in press). Interestingly, Hoson et al. (2009) reported that the ossification sequence of *Trichechus* cranial sutures is also anomalous. In the Florida manatee, sutures of the basicranium ossify after those of the face and calvarium, and in an anterior to posterior sequence. The delayed synchondrosis of the basioccipital and the exoccipital is not shared with dugongs, and Hoson et al. (2009) suggest that it may be related to the unique continuous pattern of molar replacement in manatees. The late ossification of the manatee atlas and axis centra, which lie immediately posterior to and in sequence with the basioccipital, may be another manifestation of this character suite.

Hypothesis 3. Local homeosis at the cervical/thoracic boundary predicts a one segment anterior movement of the cervical/thoracic border, with morphological transformation of V7
from cervical to thoracic morphology, a decrease in cervical count by one, and a corresponding increase in thoracic count. The presence of a moveable articulation with a rib with a sternal articulation and a centrum with thoracic ossification pattern clearly identify V7 as thoracic. Additionally, the brachial plexus occurs in the same relationship to the cervical/thoracic boundary as it does in other mammals, indicating a coordinated repatterning of abaxial as well as primaxial components in axial position 7. Although these observations support local homeosis of the cervical/thoracic border, comparisons with Dugong cervical anatomy do not. Surprisingly, both gross and quantitative analyses of shape indicate that the V7 anatomy seen in traditionally patterned Dugong is not absent. Rather, Dugong V7 anatomical features, such as the dorsal location of the transverse process base, the loss or fusion of the ventral bar, and the reduction or loss of the transverse foramen have been displaced anteriorly to axial position 6 in Trichechus. Parallel anterior displacements of both V6 and V5 Dugong anatomy to axial positions 5 and 4 in Trichechus occur as well. These features are not predicted by local homeosis. Instead, reduction in cervical count relative to Dugong appears to reflect a compression of existing cervical patterning subunits into a smaller vertebral count.

Proposal of an alternative hypothesis
Serially homologous structures display characteristic patterns of variation (Bateson 1894) that are commonly understood as the products of a small suite of developmental changes. These patterns include changes in size or shape of module members (homologous changes), changes in total count (meristic changes), changes in boundaries of adjacent submodules with reciprocal changes in count (homeotic changes), and changes in developmental architecture (associational changes). Among these options, the reduction in Trichechus cervical count from seven to six superficially resembles a local homeotic shift. However, our data suggest that a more global developmental change has occurred. C7
anatomy has not been replaced by T1 anatomy as predicted by local homeosis. Instead, C7
(as well as C6 and C5) anatomy still exists, but has been displaced anteriorly. The full range
of cervical anatomy seen in dugongs also exists in manatees, but extends over six instead
of seven vertebrae, suggesting a dissociation of axial patterning and segmentation.

Dissociation of segmentation from axial patterning has been postulated previously on
theoretical grounds (Needham 1933, Raff 1996). Although rarely documented, dissociative
events can also be inferred on the basis of patterns of variation in the axial skeletons of
multiple taxa, including mammals. For example, dissociation is implicit in the radically
different counts of delphinid cetaceans with conserved body proportions (Buchholtz 2007).
Gomez et al. (2008) also demonstrated that segmentation rates vary relative to axial growth
rate in different model vertebrates. Segment/patterning dissociation has also been
generated experimentally. Schröter and Oates (2010) disrupted the hes6 gene, which is
involved in the rate of the segmentation clock in zebrafish. They observed a decrease in the
rate of somitogenesis in mutants relative to controls, with a resulting reduction in total somite
number that was proportionately distributed across different axial regions. Mutants showed
increased segment length, but no change in axial length. Schröter and Oates (2010) further
examined anatomical markers and found that in mutants, the markers aligned with body
length and not with segment number relative to controls. They concluded that the
segmentation clock can be slowed independently from axial growth and that the
specification of axial identity occurs independently of the number of segments.

We propose that the reduction in cervical count in manatees has occurred by global
dissociation of the processes of somitogenesis and axial patterning. This hypothesis
predicts that relationships among morphological systems (vertebral, costal, sternal, neural)
have been retained, but have been globally displaced relative to somite boundaries.
Although the cervical presomitic mesoderm has been subdivided into a smaller number of larger segments, this need not result in a smaller number of cervical premuscle cells available to muscularize the diaphragm, avoiding negative selection effects on respiratory and locomotor functions.

The anterior displacement of cervical morphology observed here is consistent with the dissociation hypothesis. As a preliminary test, we compared vertebral counts and axial proportions in the postcrania of *Trichechus* relative to *Dugong*. We found smaller counts in all vertebral series (cervical, T/L or T/L/S, and caudal) of *Trichechus*, with proportionate count reductions across all axial regions. These column-wide morphological effects suggest a global, instead of a local, mechanism. Somewhat surprisingly, the fraction of postcranial length represented by the cervical, T/L or T/L/S, and caudal series was statistically identical in the two species, despite the reductions in count. These results reinforce the argument for a globally acting developmental mechanism, and coincidently raise questions about the selective pressures that may have led to its adoption.

The similarity of the proportions of neck, dorsum, and tail in *Trichechus* and *Dugong* argue against the interpretation of the reduced manatee cervical count as a response to selection for a shorter neck. Alternatively, these shared proportions may be interpreted as optimal for both species, with selection acting to reduce count throughout the postcranium of *Trichechus* via a developmental mechanism that retained these proportions. Stable positioning and hydrostatic control may have been driving forces (e.g. Domning 2000) in this selection, as a more rigid postcranium composed of a smaller number of longer vertebrae may be adaptively advantageous during prolonged shallow water harvesting of plant matter.
The dissociative hypothesis also suggests that the options for the adaptive evolution of serially homologous series are broader than commonly recognized. Highly differentiated series may be resistant to developmental innovations that impact submodule patterning, but other mutations that do not disrupt submodule integrity are apparently also available.

Dissociative mutations may be more frequently generated and/or more easily incorporated in some taxa, such as secondarily limbless tetrapods, with limited integration of somitic (primaxial) and lateral plate (abaxial) mesodermal tissues across the lateral somitic frontier (Burke and Nowicki 2003). Current morphological and developmental evidence indicates that the nontraditional cervical counts of the tree sloths Bradypus and Choloepus, and of the manatee Trichechus, have been achieved independently through different developmental processes that retain cervical patterning integrity.

ACKNOWLEDGEMENTS

We gratefully acknowledge the access to osteological specimens provided by the curatorial staffs at the American Museum of Natural History, the National Museum of Natural History, the Museum of Comparative Zoology, the Natural History Museum (London), and the University of Florida. We thank Martine deWit, Brian Bassett, and Andy Garrett of the Marine Mammal Pathobiology Laboratory, St. Petersburg, FL, and of Monica Farris of the U. S. Fish and Wildlife Service, for help in securing access to the Trichechus fetuses. Daniel VanderHart of the University of Florida College of Veterinary Medicine performed the CT scans. We thank Michael Walsh and Alex Costidis of the University of Florida College of Veterinary Medicine for access to the Trichechus neonate scan, and Darlene Ketten of the Computerized Scanning and Imaging Facility of the Woods Hole Oceanographic Institution for access to the fetal Dugong scan. Katrina Jones generously advised us on the use of
morphometrics software, and H. Grady Bailin, Sanam Anwar, Susan Laves, and Adriana Rathjen all helped with data collection and analysis.
REFERENCES


Table 1. Specimens examined in this study. § = complete postcranial skeleton; §§ = nearly complete postcranial skeleton.

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Neonate T. manatus

UF uncat.

Fetal T. manatus

MMPL M-82-5

Dusisiren jordani

UCMP 77037§
Table II. Vertebral counts and postcranial proportions in *Trichechus manatus* and *Dugong dugon* skeletons. Means are ± 1 SD.

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**Fraction of total centrum length**

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Figure Captions

Fig. 1. Cervical and T1 vertebrae of *Trichechus manatus* and *Dugong dugon* in anterior view.

A-D, *T. manatus* (A, USNM 551672; B, USNM 530315; C, USNM 551661; D, USNM 530311).

E-H, *Dugong dugon* (E, BMNH 1966.9.7.1; F, AMNH 89005; G, AMNH 105230; H, AMNH 104407). Vertebral positions are indicated below. V = vertebra. Scale = 5cm.

Fig. 2. Details of cervical anatomy in *Trichechus manatus*. A-C, unusual cervical anatomy in anterior (left) and posterior (right) views. A, USNM 527909, unilateral V5 transverse process anatomy typical of V6 (blue markers). B, USNM 530294, V6 posterior facets for the R1 capitulum (green markers) and closure of the transverse foramina with a proximal riblet (red markers); C, USNM 551681, V6 bilateral posterior facets for the TR1 capitulum (green markers) and unilateral facet for the TR1 tuberculum (white marker). D, USNM 217259, articulated cervical vertebrae in ventral view showing TR1 articulation with the posterior surface of V6. C = cervical; T = thoracic; TR = thoracic rib. Both scales = 5cm.

Fig. 3. Quantitative analysis of shape in cervical vertebrae of *Trichechus* (O) and *Dugong* (∆).

A, the preliminary PCA analysis of C1-T1 groups all C1 vertebrae from both species, all C2 vertebrae, and all post-axis vertebrae. B, the more detailed analysis of C3-T1 separates vertebrae in axial sequence on PC1, and manatees from dugongs on PC2. Colored bars identify the range of vertebrae at each axial position. Mid and posterior cervical vertebrae of manatees are more “thoracic” in shape than those of dugongs.

Fig. 4. Ossification sequence of vertebral centra and neural arches in fetal *Trichechus manatus*. A, MMPL 189 (TL = 8.4cm); B, MMPL 428 (TL = 12.9 cm); C, MMPL 9935 (TL =
21.5cm); D, MMPW 1255 (TL = 21.5cm); E, MMPL 1290 (TL = 27.7cm); F, MMPL 574 (TL = 30.0cm).

Fig. 5. Sternal ontogeny in *Trichechus* (A-H) and *Dugong* (I, J). **A-D**, fetal manatee sterna: **A**, MMPL 9935 (TL = 21.5cm); **B**, MMPL 0547 (TL = 16.0cm); **C** and **D**, surface rendered and maximum intensity projection images of MMPL M-82-5 (TL = 33.8cm). **E-H**, postnatal manatee sterna: **E**, UF uncatalogued neonate (TL = 95cm); **F** USNM 550324 (TL = 147cm); **G** USNM 550328 (TL = 249cm); **H**, AMNH 91096 (TL = 325cm). **I-J**, dugong sterna: **I**, WHOI uncat fetus (est. TL = 72 cm); **J**, AMNH 89005 adult (TL unknown), with red arrow indicating sternal suture line. Scale bars **A-D**, I = 1cm; **E-H**, J = 2cm.
Fig. 3
Fig.
4
Fig. 5