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SHORT COMMUNICATION

Humeral loads during swimming and walking in turtles: implications for morphological change during aquatic reinvasions

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ABSTRACT

During evolutionary reinvasions of water by terrestrial vertebrates, ancestrally tubular limb bones often flatten to form flippers. Differences in skeletal loading between land and water might have facilitated such changes. In turtles, femoral shear strains are significantly lower during swimming than during walking, potentially allowing a release from loads favoring tubular shafts. However, flipper-like morphology in specialized tetrapod swimmers is most accentuated in the forelimbs. To test whether the forelimbs of turtles also experience reduced torsional loading in water, we compared strains on the humerus of river cooters (*Pseudemys concinna*) between swimming and terrestrial walking. We found that humeral shear strains are also lower during swimming than during terrestrial walking; however, this appears to relate to a reduction in overall strain magnitude, rather than a specific reduction in twisting. These results indicate that shear strains show similar reductions between swimming and walking for forelimb and hindlimb, but these reductions are produced through different mechanisms.

KEY WORDS: Biomechanics, Bone strain, *Pseudemys concinna*, Evolution, Locomotion

INTRODUCTION

Habitat transitions have driven evolutionary change in many vertebrate lineages, often leading to specialization for novel environments and radiation of species (Ashley-Ross et al., 2013; Blob et al., 2016). Several ancestrally terrestrial tetrapod lineages (e.g. cetaceans, mosasaurs, manatees, sea turtles) have evolved fully aquatic lifestyles characterized by changes in body and limb shape (Zimmer, 1999; Caldwell, 2002; Lindgren et al., 2011). For example, terrestrial tetrapods have limb bones that are tubular in cross-section, shapes that help to optimize resistance to twisting (Buckwalter et al., 1995; Vogel, 2013; Blob et al., 2014); in contrast, many tetrapods that become secondarily specialized for aquatic environments exhibit flattening of the limbs (Zimmer, 1999; Renous et al., 2008). Such shapes are advantageous for producing both drag- and lift-based thrust during swimming once they are established (Walker, 2002), but the factors that promoted evolutionary transitions from tubular to flattened limbs are less clear.

Because the shapes of bones are known to respond to changes in loading environment over both ontogenetic and evolutionary time scales (Lanyon et al., 1982; Bertram and Biewener, 1990), and because buoyancy conveyed by water should reduce the loads placed on the skeleton to support the body (Zug, 1971), we previously proposed that changes in limb bone loading between land and water might have facilitated the evolution of flattened limbs in secondarily aquatic tetrapods (Young and Blob, 2015). Specifically, because torsional loading is high in the limb bones of many tetrapods (Biewener and Dial, 1995; Blob and Biewener, 1999; Butcher et al., 2008; Sheffield et al., 2011), and tubular shapes are well suited to resist torsion (Vogel, 2013), we proposed that a reduction of torsion in particular could have released the limbs from an environment favoring tubular bones and, thereby, facilitated the evolution of flattened shapes (Young and Blob, 2015). To test this proposal, we compared *in vivo* bone strains between terrestrial walking and swimming for the femur of semi-aquatic slider turtles, *Trachemys scripta* (Young and Blob, 2015). Turtles are advantageous models for these comparisons because the fusion of the vertebrae to the shell means that propulsion is generated exclusively by the limbs, and comparisons between environments are not confounded by shifts between axial and appendicular propulsion (Gillis and Blob, 2001). Our choice of a semi-aquatic species as a model reflected its use of rowing limb movements, which were also likely used by species in the initial stages of aquatic reinvasions (Fish, 1996). Moreover, our focus on the femur reflected the dominant propulsive role of the hindlimb in semi-aquatic turtles (Blob et al., 2008). Our results showed that torsional shear strains on turtle femora did, in fact, decrease much more than bending strains between terrestrial walking and swimming (Young and Blob, 2015). These results were due partly to an overall decrease in load magnitude in water. However, they also resulted from a substantial change in loading regime, in which principal strains became reoriented to align much more closely with the long axis of the femur during swimming (6.1 deg) than during walking (19.8 deg). These patterns indicated sharply reduced twisting of the femur about its long axis during swimming, a conclusion that was verified by subsequent XROMM observations of femoral kinematics in turtles (Mayerl et al., 2016).

Although strain data from turtle femora indicate that reduced torsional loads during aquatic locomotion could have generated a mechanical environment favorable for the evolution of non-tubular limb bones, the restriction of these data to the femur is problematic. In most lineages of tetrapods that became secondarily specialized for aquatic locomotion, including sea turtles, the forelimbs come to dominate appendicular-based propulsion (Wyneken, 1997; Blob et al., 2016). Thus, if changes in loading are to provide a plausible mechanism that could have contributed to the evolution of flattened limbs during aquatic reinvasions, then a reduction in torsion during swimming should be found in the humerus as well as the femur. However, no loading data are available for the forelimbs of any turtle, or any swimming tetrapod. To test whether loading patterns differ between terrestrial walking and swimming for the forelimb, we collected *in vivo* humeral strain data from semi-aquatic river cooter turtles, *Pseudemys concinna* (LeConte 1830), a species that

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is closely related and ecologically similar to *T. scripta* (Ernst and Lovich, 2009), but which reaches larger body sizes that facilitate strain gauge implantation onto the humerus. If the humerus does not show reduced torsion during swimming in turtles, then the plausibility of limb bone flattening having been facilitated by environmental changes in loading regime would be called into question.

MATERIALS AND METHODS

Animals

Six adult *P. concinna* (3 females, 3 males; carapace length 28.15±2.46 cm; mass 2.65±0.61 kg) were collected from Lake Hartwell, Pickens County, SC, USA (August 2013 and August 2014, South Carolina Department of Natural Resources Permits 43-2013, 29-2014). Housing and husbandry followed published standards (Butcher et al., 2008).

Surgical procedures

All procedures were approved by the Clemson University IACUC (AUP 2012-056, 2016-011). To induce analgesia and anesthesia, turtles were injected (left forelimb muscles) with doses of 1 mg kg^{-1} butorphenol, 100 mg kg^{-1} ketamine and 1 mg kg^{-1} xylazine (supplemented as needed). Upon achieving anesthesia, a medial incision was made along the proximal aspect of the right forelimb. Muscles surrounding the humerus were separated and retracted to expose gauge attachment sites. A window of periosteum was removed, and the exposed bone cortex was swabbed clean with ether. Single-element and rosette strain gauges (FLG-1-11 and FRA-1-11, respectively; Tokyo Sokki Kenkyujo, Japan) were attached using self-catalyzing adhesive (Duro® Super Glue; Henkel Corporation, Avon, OH, USA). In our largest individual, we implanted a rosette gauge on both the anterior and posterior surface of the humerus. In other large individuals, we attached a rosette gauge to either the anterior or ventral surface, and single-element gauges to two other surfaces (anterior, ventral or posterior). For our smallest individuals, in which rosette gauges could not be implanted because of size limitations, three single-element gauges were attached in anterior, ventral and posterior positions. Once gauges were in place, lead wires were threaded through a second, proximal forelimb incision. Incisions were sutured closed and wires were soldered to a microconnector and sealed with epoxy. Connectors were secured to the forelimb with self-adhesive bandage (Vetrap®; 3M Animal Care Products, Maplewood, MN, USA), with care taken to avoid restricting limb movement.

In vivo strain data collection and data analysis

Following 24 h of recovery, *in vivo* strain data were collected during steady-speed swimming in a flow tank and walking on a motorized treadmill (model DC5; Jog A Dog®, Ottowa Lake, MI, USA). Strain signals were conducted from the gauges to Vishay conditioning bridge amplifiers (model 2120B; Measurements Group, Raleigh, NC, USA) by a shielded cable. To prevent signal disruption by water, the connection between this cable and the connector attached to the turtle was sealed with Plumber's Epoxy Putty (ACE Hardware Corporation, Oak Brook, IL, USA). Raw voltages from strain gauges were sampled through an A/D converter (model PCI-6031E; National Instruments, Austin, TX, USA) at 5000 Hz. These data were saved to computer using data acquisition software (LabVIEW v.6.1; National Instruments) and calibrated to microstrain (με).

Trials were conducted at the maximal speed at which an individual could maintain its position in the flow tank or on the treadmill (0.200–0.495 m s^{-1} in a flow tank; 0.103–0.139 m s^{-1} on a treadmill). Although these speeds are not strictly dynamically equivalent, they provide comparable levels of exertion that are useful for understanding selection pressures acting on skeletal design. High-speed videos of each trial were recorded from lateral and ventral (swimming) or dorsal (walking) views (100 Hz; Phantom v5.1, Vision Research Inc., Wayne, NJ, USA). Videos were synchronized with strain recordings using a light box that emitted a visible flash in the video that corresponded with a 1.5 V pulse in the strain recording. Strain recordings were zeroed by selecting samples of 100 values from each trial during intervals of limited motion (swimming) or resting with the shell on the treadmill (walking). Upon completion of trials, turtles were euthanized via intraperitoneal injection of Euthasol® pentobarbital sodium solution (200 mg kg^{-1}; Delmarva Laboratories Inc., Midlothian, VA, USA).

Because of gauge failure at some sites, particularly during aquatic trials, strain data were only collected from a subset of the locations at which gauges were initially implanted. As a result, longitudinal strain data were collected from the posterior surface of the humerus for three individuals, the ventral surface for three individuals and the anterior surface for one individual. Swimming principal strains were collected from three individuals, and terrestrial walking principal strains were collected from two individuals (Tables 1 and 2). Peak strain magnitudes were determined from each functioning gauge location for each stroke (swimming) and step (walking) of the right forelimb, following previously published methods (Blob and Biewener, 1999). Walking and swimming strains were compared within each individual for each gauge location using Mann–Whitney *U*-tests. Statistical analyses were conducted in SAS® (v.9.3, SAS Institute Inc. 2010, Cary, NC, USA).

RESULTS AND DISCUSSION

During swimming, longitudinal strains generally maintained the same orientation (i.e. tensile or compressive) during both thrust (retraction) and recovery (protraction) phases of the limb cycle for four out of seven comparisons (Table 1). Thus, the humerus showed reversals in its direction of bending between protraction and retraction more frequently than the femur (Young and Blob, 2015). Single peaks were typically observed during retraction in swimming, whereas strains were more variable during protraction (Fig. 1), resembling patterns observed in the femur (Butcher et al., 2008; Young and Blob, 2015). In contrast to the femur (Young and Blob, 2015), absolute magnitudes of peak humeral strain during swimming (longitudinal, principal and shear) were not uniformly greater during thrust than during recovery (Table 1). These differences between humeral and femoral loading may reflect differences in the size of the paddle formed by the foot in each limb. In both limbs, the foot is rotated perpendicular to oncoming flow during retraction (Pace et al., 2001; Blob et al., 2008), maximizing surface area of the foot against the surrounding medium to produce drag-based thrust. During the recovery phase (protraction), the foot is rotated parallel to oncoming flow, reducing drag and minimizing interference to forward motion of the body. Such drag reduction is expected to minimize the environmental forces acting on the limb, resulting in lower strains during recovery (Young and Blob, 2015). However, the surface area of the forefoot paddle is much smaller than the surface area of the hindfoot paddle in cooters and sliders (Young et al., 2017a), which may lead to greater similarity in the environmental forces applied to the limb between thrust and recovery phases for the forelimb. Moreover, orientation of peak principal tensile strain to the long axis of the humerus (ϕ_T) was typically near 45 deg during both thrust and recovery, indicating the significance of twisting as a mechanism through which loads are

Table 1. Mann–Whitney *U*-test results for comparisons of peak humeral strain during thrust versus recovery phases of swimming in *Pseudemys concinna*

ID	Gauge location	Strain type	*N*	Thrust (με)	Recovery (με)	|*Z*|	*P*
PC01	Posterior	Longitudinal	40	58.1±11.9	−60.7±18.8	3.30	**0.0010**
PC02	Ventral	Longitudinal	71	−19.6±12.6	−89.4±10.6	3.22	**0.0013**
	Posterior	Longitudinal	71	−13.1±18.7	−88.1±16.2	2.75	**0.0059**
PC03	Posterior	Longitudinal	22	36.9±24.2	−83.7±27.5	2.27	**0.0235**
PC04	Anterior	Longitudinal	34	32.7±13.2	8.7±12.7	1.05	0.2943
	Anterior	pT	34	72.2±7.3	63.7±7.4	0.98	0.3295
	Anterior	pC	34	−70.5±5.8	−73.5±7.0	0.35	0.7267
	Anterior	ϕ_T	34	51.8±4.1	42.9±4.6	1.21	0.2270
	Anterior	Shear	34	93.8±11.4	90.1±13.8	0.52	0.6022
PC05	Ventral	Longitudinal	12	156.9±35.6	55.2±73.0	0.09	0.9310
	Ventral	pT	12	193.6±26.4	155.9±52.7	2.11	**0.0351**
	Ventral	pC	12	−184.2±32.2	−215.2±49.1	0.20	0.8399
	Ventral	ϕ_T	12	24.1±7.5	48.2±11.7	0.49	0.6236
	Ventral	Shear	12	137.0±24.5	50.4±11.0	2.68	**0.0073**
PC06	Ventral	Longitudinal	85	51.6±7.5	−87.3±3.7	2.08	**0.0376**
	Ventral	pT	85	156.2±10.9	54.9±6.7	8.46	**<0.0001**
	Ventral	pC	85	−124.9±11.5	−117.7±0.1	0.22	0.8273
	Ventral	ϕ_T	85	36.3±2.0	42.6±3.6	0.24	0.8104
	Ventral	Shear	85	242.4±23.1	93.7±16.7	6.48	**<0.0001**

Values are means±s.e.m.; pT, principal tensile strain; pC, principal compressive strain; ϕ_T, angle (deg) of principal tensile strain to the humeral long axis; με, microstrain. Bold indicates significance.

applied to the forelimb (Table 1). These orientations are a further departure from the patterns observed in the femur, in which ϕ_T was closer to 0 deg during both thrust and recovery.

In comparisons between swimming and walking, the orientation of longitudinal strains on the humerus was typically consistent between environments (four out of six comparisons; Table 2). Peak strain magnitudes also were consistently significantly lower during the thrust phase of swimming than during the stance phase of walking (Table 2, Fig. 1). For longitudinal strains during retraction, peak magnitudes during swimming were approximately 11% of peak magnitudes during walking. For shear, however, peak swimming strain magnitudes were roughly 40% of walking strains (Table 2, Fig. 1). Though this is a considerable reduction in load between locomotor environments, it is less of a reduction in shear between environments than was found for the femur, in which shear strains during swimming were only 10% of those during walking (Young and Blob, 2015). In the femur, shear strain reduction during swimming is driven by both an overall reduction in strain magnitudes conveyed by buoyancy in water, and a reorientation of loading that reduces the high levels of twisting observed in walking to lower levels during swimming (Young and Blob, 2015; Mayerl et al., 2016). In contrast, the reduction of humeral shear strains during swimming appears to result essentially solely from the overall reduction of strain magnitudes in water compared with land (Table 2). Values of ϕ_T for the humerus (Table 2) were substantially greater than 0 deg during both terrestrial walking and swimming, indicating that twisting is likely applied to the humerus in both environments. Therefore, though both shear and torsional loading on the humerus are reduced during swimming compared with walking, this reduction does not appear to result from the substantial reorientation of applied loads that occurs in the femur.

The different mechanisms that reduce aquatic shear strains in the humerus versus the femur of turtles may relate to structural differences between the forelimb and hindlimb, and the kinematic constraints that these impose. The extent of forelimb protraction in turtles is unusually high for tetrapods with sprawling postures (Walker, 1971; Pace et al., 2001; Schmidt et al., 2016). Such protraction may be facilitated by humeral morphology, particularly its arched shaft and the anatomical torsion of the distal humerus

Table 2. Mann–Whitney *U*-test results for comparisons of peak humeral strain during swimming versus terrestrial walking for the thrust/stance phase of the limb cycle in *P. concinna*

ID	Gauge location	Strain type	*N* (swim; walk)	Swim (με)	Walk (με)	|*Z*|	*P*
PC01	Posterior	Longitudinal	40; 35	58.1±11.9	1398.9±37.6	7.43	**<0.0001**
PC02	Ventral	Longitudinal	71; 28	−19.6±12.6	251.9±64.1	7.58	**<0.0001**
	Posterior	Longitudinal	71; 28	−13.1±18.7	277.2±58.6	7.28	**<0.0001**
PC03	Posterior	Longitudinal	22; 8	36.9±24.2	746.4±95.5	4.10	**<0.0001**
PC05	Ventral	Longitudinal	12; 29	156.9±35.6	562.5±93.8	4.97	**<0.0001**
	Ventral	pT	12; 29	193.6±26.4	699.3±46.1	3.97	**<0.0001**
	Ventral	pC	12; 29	−184.2±32.2	−249.5±32.1	2.22	**0.0264**
	Ventral	ϕ_T	12; 29	24.1±7.5	13.9±3.7	1.42	0.1561
	Ventral	Shear	12; 29	137.0±24.5	285.5±30.7	2.97	**0.0030**
PC06	Ventral	Longitudinal	85; 32	51.6±7.5	274.2±56.1	8.30	**<0.0001**
	Ventral	pT	85; 32	156.2±10.9	556.2±31.8	7.88	**<0.0001**
	Ventral	pC	85; 32	−124.9±11.5	−362.2±32.4	6.37	**<0.0001**
	Ventral	ϕ_T	85; 32	36.3±2.0	56.7±3.1	4.98	**<0.0001**
	Ventral	Shear	85; 32	242.4±23.1	729.2±56.5	6.30	**<0.0001**

Values are means±s.e.m. pT, principal tensile strain; pC, principal compressive strain; ϕ_T, angle (deg) of principal tensile strain to the humeral long axis; με, microstrain. Bold indicates significance.

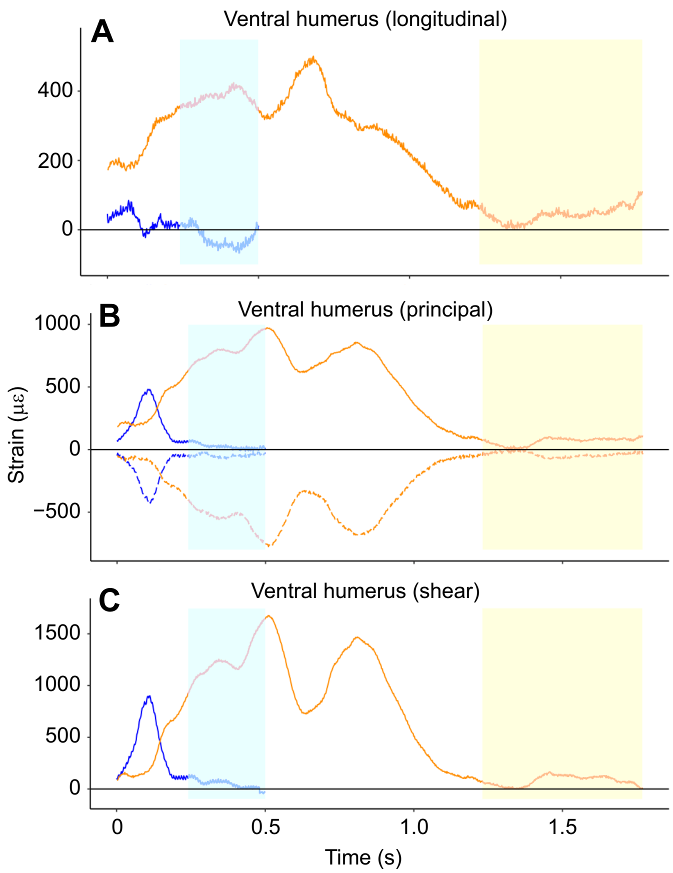

Fig. 1. Strain traces from the forelimb of a river cooter turtle (*Pseudemys concinna*). Examples of high-magnitude strain traces from turtle PC06, simultaneously recorded from a rosette gauge located on the ventral surface of the humerus during swimming and terrestrial walking. A single limb cycle from the same individual is illustrated for both behaviors. (A) Ventral longitudinal strain. (B) Ventral principal strain. (C) Ventral shear strain. Walking strains are shown in orange and swimming strains are shown in blue. Shaded regions indicate the recovery (protraction) phase of the limb cycle for each locomotor behavior (walking in yellow, swimming in light blue). Compressive principal strain is represented by a dashed line. Note the duration of the swimming cycle is approximately half that of the walking cycle. με, microstrain.

relative to the head (Ogushi, 1911). However, humeral retraction in turtles is generally limited (Rivera and Blob, 2010; Schmidt et al., 2016), likely as a result of restrictions imposed by the anterior edge of the bridge between the carapace and plastron (Walker, 1971; Zug, 1971). As a potential consequence, in tortoises walking on land, the majority of forelimb range of motion (64%) is derived from long-axis rotation combined with elbow extension (Schmidt et al., 2016). Given the large potential impact of axial rotation on the range of forelimb motion, a reduction of humeral twisting in water might impose substantial locomotor restrictions on turtles. Therefore, long-axis rotation of the humerus may be necessary in both aquatic and terrestrial habitats in order to achieve adequate range of motion for forward propulsion, and could explain why strain orientations near 45 deg are observed in both habitats. Such locomotor restrictions of the humerus stand in contrast to the limited impact that reduced femoral twisting appears to have on hindlimb movements (Mayerl et al., 2016), as the hindlimbs do not experience the same degree of shell obstruction and, therefore, do not need to maintain long-axis rotation to sustain limb excursion and generate thrust in aquatic environments.

Strain patterns of the long bones of the limb indicate reduced shear during swimming compared with terrestrial walking in both the forelimb and the hindlimb (Young and Blob, 2015). Despite showing similar patterns of shear reduction, changes in loading between land and water may occur through different mechanisms in the humerus and femur that relate to structural and functional differences between the forelimb and hindlimb in turtles. Nonetheless, the distinctive changes in long-bone morphology that characterize most reinvasions of aquatic habitats by tetrapods may likely have been facilitated by release from the demands imposed by body support and torsional loading, allowing greater opportunity for the evolution of novel limb bone shapes.

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Competing interests
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Author contributions
Conceptualization: V.KH.Y., R.W.B.; Methodology: V.KH.Y., R.W.B.; Software: V.KH.Y., R.W.B.; Validation: V.KH.Y., R.W.B.; Formal analysis: V.KH.Y., C.E.W., B.P.W., R.W.B.; Investigation: V.KH.Y., C.E.W., B.P.W., R.W.B.; Resources: V.KH.Y., C.E.W., B.P.W., R.W.B.; Data curation: V.K H.Y., R.W.B.; Writing - original draft: V.KH.Y., C.E.W., B.P.W., R.W.B.; Writing - review & editing: V.KH.Y., C.E.W., B.P.W., R.W.B.; Visualization: V.KH.Y., R.W.B.; Supervision: V.KH.Y., R.W.B.; Project administration: V.KH.Y., R.W.B.; Funding acquisition: R.W.B.

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Data availability
Data are available from the Dryad Digital Repository (Young et al., 2017b): doi:10.5061/dryad.tv7fk

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