Locomotor plasticity of an amphibious fish (*Polypterus senegalus*)

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ABSTRACT

Animals control locomotion through unpredictable and complex habitats using a single locomotor control system. Because of the disparate physical mechanics of different environments, behavioural plasticity, based on the complex interplay of sensory feedback and environmental constraints, is likely essential for animals moving across environments. However, few studies have investigated neuromuscular control across different environments. To fill this gap, I make use of *Polypterus senegalus* to address four primary objectives: (1) to explore the extent of neuromuscular plasticity across environmental gradients (viscosity and water depth), (2) to generate and test hypotheses about paramount signals for this neuromuscular plasticity, (3) to determine the neuromuscular underpinnings of locomotor transitions, and (4) to determine the neuromuscular control of developmental behavioural plasticity in novel environments. I measured the kinematic and muscle activity response of *P. senegalus* to gradual changes in environment forces using gradients of water viscosity and water depth. I then used a semi-intact preparation to investigate the existence and role of the mesencephalic locomotor region, a brain region that controls locomotor speed and mode in other species, for neuromuscular control in *P. senegalus*. Finally, I used chronic terrestrial acclimation and exercise to determine the neuromuscular underpinnings of behavioural and morphological plasticity previously seen in *P. senegalus* reared in a terrestrial environment. I found that in high viscosity environments, *P. senegalus* maintain routine swimming speed using a swimming-like muscle activity pattern with increased effort in the posterior body and the pectoral fin to generate exaggerated swimming kinematics. These results suggest that sensory feedback is essential to accommodating this novel environment. I then demonstrated that axial red muscle always carried an anterior-to-posterior wave of muscle activity in a series of discrete water depths across the aquatic-terrestrial transition. Thus, discrete changes in axial kinematics and pectoral fin coordination across this transtion are likely the result of sensory feedback and mechanical constraints of the

environment. I then performed the first experiments searching for the mesencephalic locomotor region in *P. senegalus* and demonstrated the presence of a putative mesencephalic locomotor region that controls the frequency of swimming-like movements but does not appear to control pectoral fin movements or the transition to walking. Finally, I exposed *P. senegalus* to chronic terrestrial acclimation and exercise. My results suggested that while both terrestrial acclimation and exercise generate behavioural plasticity, the former results in a larger plastic repsonse. Subtle changes in the duration and timing of pectoral fin muscle activity helped reduce friction between the body and pectoral fin and the substrate below, potentially resulting in the more "effective" walking gait developed by terrestrial acclimated fish. My thesis therefore sheds light on the essential interplay of sensory feedback and mechanical constraint for generating behavioural plasticity on acute and chronic timescales, highlights the potential value of such plasticity for organismal performance and evolution, and develops study systems and experimental frameworks for further investigating the nature of plastic locomotor control in amphibious fish.

RÉSUMÉ

Les animaux contrôlent leur locomotion dans des habitats imprévisibles et complexes à l'aide d'un seul système de contrôle locomoteur. En raison de la mécanique physique disparate des différents environnements, la plasticité comportementale, basée sur l'interaction complexe du feedback sensoriel et des contraintes environnementales, est probablement essentielle pour que les animaux se déplacent à travers les environnements. Cependant, peu d'études ont étudié le contrôle neuromusculaire dans différents environnements. Pour combler cette lacune, j'utilise *Polypterus senegalus* pour répondre à quatre objectifs principaux : (1) explorer l'étendue de la plasticité neuromusculaire à travers des gradients environnementaux (viscosité et profondeur de l'eau), (2) générer et tester des hypothèses sur les signaux primordiaux pour cette plasticité neuromusculaire, (3) déterminer les fondements neuromusculaires des transitions locomotrices, et (4) déterminer le contrôle neuromusculaire de la plasticité comportementale de développement dans de nouveaux environnements. En créant des gradients de viscosité et de profondeur d'eau, j'ai examiné les changements de cinématique et d'activité musculaire de *P. senegalus* en réponse à des changements graduels des forces environnementales. J'ai ensuite utilisé une préparation semi-intacte pour étudier l'existence et le rôle de la région locomotrice mésencéphalique, une région du cerveau qui contrôle la vitesse et le mode locomoteurs chez d'autres espèces, pour le contrôle neuromusculaire chez *P. senegalus*. Enfin, j'ai utilisé l'acclimatation terrestre chronique et l'exercice pour déterminer les fondements neuromusculaires de la plasticité comportementale et morphologique précédemment observée chez *P. senegalus* élevé dans un environnement terrestre. J'ai découvert que dans des environnements à haute viscosité, *P. senegalus* maintient sa vitesse de nage habituelle en utilisant un modèle d'activité musculaire semblable à celui de la natation avec un effort accru dans la partie postérieure du corps et la nageoire pectorale pour générer une cinématique de nage exagérée. Ces résultats suggèrent que la rétroaction sensorielle est essentielle pour s'adapter à ce nouvel

environnement. J'ai ensuite démontré que le muscle rouge axial était toujours porteur d'une vague d'activité musculaire antérieure à postérieure dans une série de profondeurs d'eau discrètes à travers la transition aquatique-terrestre. Ainsi, les changements discrets de la cinématique axiale et de la coordination de la nageoire pectorale au cours de cette transition sont probablement le résultat de la rétroaction sensorielle et des contraintes mécaniques de l'environnement. J'ai ensuite réalisé les premières expériences de recherche de la région locomotrice mésencéphalique chez *P. senegalus* et j'ai démontré la présence d'une région locomotrice mésencéphalique putative qui contrôle la fréquence des mouvements de type natation mais qui ne semble pas contrôler les mouvements de la nageoire pectorale ou la transition vers la marche. Enfin, j'ai exposé *P. senegalus* à une acclimatation terrestre chronique et à l'exercice. Mes résultats suggèrent que si l'acclimatation terrestre et l'exercice génèrent tous deux une plasticité comportementale, la première entraîne une réponse plastique plus importante. Des changements subtils dans la durée et le moment de l'activité musculaire de la nageoire pectorale ont contribué à réduire la friction entre le corps/la nageoire pectorale et le substrat en dessous, ce qui a potentiellement conduit à la démarche de marche plus "efficace" développée par les poissons acclimatés terrestres. Ma thèse met donc en lumière l'interaction essentielle entre le retour sensoriel et la contrainte mécanique pour générer une plasticité comportementale sur des échelles de temps aiguës et chroniques, souligne la valeur potentielle d'une telle plasticité pour la performance et l'évolution de l'organisme, et développe des systèmes d'étude et des cadres expérimentaux pour approfondir la nature du contrôle locomoteur plastique chez les poissons amphibies.

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We pay respect to the Algonquin people, who are the traditional guardians of this land. We acknowledge their longstanding relationship with this territory, which remains unceded. We pay respect to all Indigenous people in this region, from all nations across Canada, who call Ottawa home. We acknowledge the traditional knowledge keepers, both young and old. And we honour their courageous leaders: past, present, and future.

PREFACE

This thesis consists of four data chapters (chapters 2 – 5), each of which are prepared in manuscript style. Preceding each chapter is a description where the manuscript is published and/or where in the review process the manuscript is, a list of author contributions, and specific acknowledgements for each chapter. This thesis was supported by The Natural Sciences and Engineering Research Council of Canada [NSERC Discovery Grant to Emily M. Standen] and was additionally funded through the Human Frontiers Science Program [RGP0027/2017 to Emily M. Standen, Auke J. Ijspeert, and Akio Ishiguro]. I was a holder of an Ontario Graduate Scholarship for four years during this thesis.

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GLOSSARY AND ABBREVIATIONS

Activational behavioural plasticity: short-term behavioural plasticity based on the differential recruitment of existing neural networks.

aCSF: artificial cerebrospinal fluid

ANOVA: analysis of variance

BD: body depth. Measured from the ventral aspect to the dorsal aspect of the deepest part of the fish. Does not include the dorsal fin.

BL: body length, measured as total length.

ChAT: choline acetyltransferase

CPG: central pattern generator, a network of interconnected spinal neurons capable of producing rhythmic motor output in isolation of sensory feedback.

Developmental behavioural plasticity: long-term behavioural plasticity based on the building of new neural networks.

Duty factor: duration of a kinematic or muscle activity event expressed as a percentage of the kinematic cycle (also known as duty cycle).

Dynamic loading: loading of bones and tissues that occurs as a result of locomotor movements.

EMG: electromyography

EMG set: for chapter 3, the set of fishes used in EMG experiments.

Gait: a discrete pattern of body and limb movements that occurs at a particular locomotor speed.

Kinematics: a description of body and limb movements.

Kinematics set: for chapter 3, the set of fishes used in kinematic experiments.

LDT: laterodorsal tegmental nucleus

MLR: mesencephalic locomotor region

MS222: tricane methanesulfonate

PBS: phosphate buffered solution

Plasticity: the ability to (reversibly or not) alter behavioural, morphological, physiological and life-history traits to suit the environment.

PPN: pedunculopontine nucleus

R^2c: conditional R-squared. An estimated R-squared for linear mixed effects models based solely on the fixed effects of the model.

R^2m: marginal R-squared. An estimated R-squared for linear mixed effects models based on both the fixed and random effects of the model.

Re: Reynold's number.

RIA: rectified integrated area. Here, used to refer to the rectified integrated area of a burst of muscle activity.

RoM: range of motion.

Static loading: loading of bones and tissues that occurs when the animal is stationary.

CHAPTER 1: GENERAL INTRODUCTION

Locomotion is the ability of an organism to move from one place to another. This capacity for movement is fundamental to the biology of a wide array of animals. For centuries, researchers have been fascinated with how animals move owing to locomotion's plastic nature, its inspiration of engineering solutions, and its role as an integrative, functional outcome of physiological systems. The control of locomotion across all levels of organization, from population-level movements (e.g. Nathan et al., 2008) to the genetic underpinnings of behavioural phenotypes (e.g. Sokolowski, 2001), has been widely studied. These investigations have led to a foundational understanding of animal movement (e.g. Dickinson et al., 2000), advances in robotics (e.g. Delmerico et al., 2019), and an appreciation for common principles that govern the control of locomotion across species (e.g. Grillner and El Manira, 2020). Among these common principles is the recognition that sensory feedback from the environment, internal mechanics of bones and muscles, and physical constraints of the environment are all integral for generating effective locomotion. However, efforts to disentangle how these factors interact during natural movement are still in their nascence (e.g. Gillis and Blob, 2001; Nishikawa et al., 2007) and/or restricted to a few taxa (e.g Grillner and El Manira, 2020). A detailed understanding of how muscles generate movement across environments and in a variety of species is critical to this effort.

1.1 Locomotor control system

Detailed descriptions of animal movement began in 1878 when Eadwaerd Muybridge used still images to determine there was an aerial phase to a horse's gallop. Since then, high-speed photography and videography have been employed countless times to describe how animals move. However, fewer studies have used locomotor movements in combination with data from muscle and nerves, to elucidate locomotor control. Investigations by Marey (1980) laid the groundwork for

recording from muscles, while Brown (1911) and von Holst (1935a,b) were among the first to hypothesize that the spinal cord alone is sufficient to generate repeated, rhythmic movements. Our current understanding suggests the rhythmic muscle contractions that generate repeated movements in animals across the phylogenetic tree are generated by a series of intrinsically rhythmic (i.e. in the absence of sensory feedback) neural circuits, each called a central pattern generator (CPG) (Cabelguen et al., 2010; Delcomyn, 1980; Goulding, 2009; Grillner, 1975; Grillner et al., 1991; Grillner and El Manira, 2020; Marder and Calabrese, 1996; Rossignol et al., 2006). The sequential firing of CPG neurons and associated interneurons dictate the activation of motor neurons. These motor neurons are directly responsible for generating muscle contractions that power locomotion. Body and limb movements (i.e. kinematics) are generated through the forces of muscular contraction, which themselves are shaped by the physical forces of the environment and the physical constraints of an animal's morphology (Biewener and Gillis, 1999; Gillis and Blob, 2001; Nishikawa et al., 2007; Tytell et al., 2011). Thus, especially as animals move between physical media, changing environmental forces may play a critical role in locomotor performance.

While CPGs are the fundamental unit of rhythmic locomotor control, they rely on sensory feedback to accommodate the heterogeneity and unpredictability of natural habitats. This sensory feedback can input directly onto spinal CPGs (e.g. proprioception - Bosco and Poppele, 2001; lamprey spinal stretch cells - Grillner et al., 1982, Grillner et al., 1984; zebrafish spinal proprioception cells – Picton et al., 2021) and/or is first integrated in higher processing centers (cortex in mammals, pallium in fish) before being passed to the spinal CPGs. In many vertebrates, integrated sensory information projects to the mesencephalic locomotor region or diencephalic locomotor region (Dubuc et al., 2008; El Manira et al., 1997). These brain regions act as rheostats turning up or down the strength of the signal (passed via reticulospinal neurons) that drives the CPGs, adjusting the frequency (Sirota et al., 2000) of movements, triggering gait transitions

(Cabelguen et al., 2003), and initiating locomotion (El Manira et al., 1997). The diversity of locomotion seen in extant animals is thus a consequence of a complex interplay between sensory feedback, the physical limitations of an animal's morphology, and the physical properties of their habitat. The notion that sensory feedback and physical constraints are critical in locomotion is well accepted. But the specific nature of their influence on behaviour has only recently begun to be studied and has largely been restricted to a few model taxa (e.g. Grillner and El Manira, 2020).

1.2 Neuromuscular plasticity following environmental changes

On a near-instantaneous basis, sensory feedback helps tune motor control to generate effective movement. This is neuromuscular plasticity on the shortest of timescales. Plasticity is commonly defined as the ability to (reversibly or not) alter behavioural, morphological, physiological, and life-history traits to suit the environment (Pfennig, 2010; West-Eberhard, 2003; Whitman and Agrawal, 2009; Wright and Turko, 2016). Plastic responses to environmental perturbations directly impact ecology and evolution by altering individual phenotype (reviewed in Pfennig, 2021). If the costs of plasticity are low, these responses may be especially pronounced for animals moving between habitats with dramatically different selective pressures (e.g. Wright and Turko, 2016). Because behavioural and muscle activity plasticity can occur over short timescales and is often reversible, it can be particularly important during responses to rapid environmental change (Pfennig, 2021). For example, behavioural plasticity can shelter species from unfavourable conditions, slowing evolutionary change, but may also open access to new niches and can even promote positive feedback loops that change habitat preferences (Crespi, 2004; Tigert et al., 2021 preprint).

The timescale over which behavioural plasticity occurs dictates the mechanism(s) that generate a change. Over short time scales (*activational* behavioural plasticity; Snell-Rood, 2013), changes in the sequence of motor nerve firing or to the pool of active motor units alter behaviour in

response to the environment (Daley and Biewener, 2003) or to generate different speeds (e.g. Jayne and Lauder, 1994). However, neural architecture changes require more time. Long-term changes (*developmental* behavioural plasticity; Snell-Rood, 2013) in environment, behavioural necessity, and injury can re-wire CPG circuits (within hours, Sakurai and Katz, 2009; or days, Barrière et al., 2008; Barrière et al., 2010; Gossard et al., 2015; Martinez et al., 2011) and also modulate the activity level of motor- and interneurons (El Manira, 2014). Since *activational* behavioural plasticity requires no changes in morphology, it is likely critical for initial responses to a new environment. If an individual spends more time in this new environment, *developmental* plasticity may fine tune behavioural changes to best suit environmental pressures.

Like neural activity and neural morphology, muscle activity pattern, muscle function, and muscle architecture are also plastic. For example, shifting muscle activity timing relative to cyclical muscle length changes, which is possible on a near-instantaneous timescale, may alter where on the force-length or force-velocity curve a muscle operates (Fig 1.1). These changes directly influence the amount of force generated by and work output of muscles, altering their function. Repeated behaviour requiring a specific muscle function and muscle force generally improves performance in that task. This is especially obvious after exercise. In both mammals and fish, aerobic exercise improves the aerobic capacity of muscle, and anaerobic exercise improves the anaerobic capacity of muscle (McClelland, 2012). This increased capacity is the result of myosin isoform expression, mitochondrial density, oxygen and nutrient delivery, and waste clearance. Therefore, changes in the function and structure of muscle impact the available scope of performance, and thus the limits for neuromuscular and behavioural plasticity.

1.3 Experimental frameworks for investigating locomotor control

Locomotor plasticity in response to the environment is most often studied at the level of behaviour. A large body of previous work investigates the behavioural plasticity of fish, reptiles, and

invertebrates in different environments including between water and land, in various arboreal habitats, and across different substrate roughness (e.g. Foster and Higham, 2012; Gibb et al., 2013; Horner and Jayne, 2008; Pace and Gibb, 2014; Standen et al., 2016; Yasui et al., 2019). The overwhelming majority of this research describes kinematic changes in response to natural environmental shifts (but see Standen et al., 2016 and Yasui et al., 2019 for examples of exceptions). Such observational data are essential to our understanding of ecology, fitness, and performance, as well as in describing differences in movement between environments. However, these studies seldom investigate the role of locomotor control in behavioural changes. What changes in sensory feedback does an amphibious fish experience on land? How might this change its capacity for effective movement? How does a fish generate the required force to lift itself off the ground? For example, the flow-sensitive lateral line system is essential to a variety of swimming functions (Bleckmann and Zelick, 2009; Coombs et al. 2001; Engelmann et al. 2000; McHenry et al. 2008), but it is unclear if this sensory system is functional for an amphibious fish on land. Thus, fish moving on land may not have the full suite of sensory systems available to them in water. Several recent review papers have highlighted the value of cross-environment studies for elucidating the function of neuromuscular control systems that generate coordinated movement in natural settings (Blob and Higham, 2014; Gillis and Blob, 2001; Lutek et al., 2022; Nishikawa et al., 2007). Building on the plethora of descriptive studies in different environments, we are now poised to address mechanistic hypotheses about the nature of neuromuscular control across environments. Starting with careful examination of kinematics and muscle activity, this thesis aims to open a window into the interplay of sensory feedback and environmental constraint.

In theory, there are three ways muscle activity and outward kinematics can change in a new environment. Each one points to a particular combination of mechanical constraint and sensory feedback as underlying mechanisms. First, if sensory feedback and mechanical constraint are

interacting to shape outward kinematics, both kinematics and muscle activity change between environments. The critical influence of mechanical constraint can be seen in two additional scenarios: (1) kinematics change, but muscle activity does not and (2) muscle activity changes, but kinematics do not. In (1) sensory feedback is not altering neuromuscular output, while in (2) sensory feedback is eliciting a change, but the environment is overriding the neuromuscular pattern. This framework allows us to interpret what neuromuscular changes facilitate behavioural plasticity in new environments.

Beyond discrete changes in environment, continuous environmental gradients may provide further insights into the neuromuscular mechanisms that underlie behavioural plasticity. In several species of fish, investigators have looked at behavioural and muscle activity changes across discrete differences in environment (e.g. terrestrial and aquatic environments, Foster et al., 2018; Gillis, 2000; Pearlman and Ashley-Ross, 2016). While these offer an essential first description of how behavioural plasticity can facilitate locomotion in dramatically different environments, comparisons of only two conditions lack the subtlety to determine whether motor control changes continuously or discretely (Fig. 1.2). If a continuous change in neuromuscular control is observed across a gradient of environmental conditions, this likely indicates a single neural circuit at play for both behaviours. An excellent example that uses this framework comes from *C. elegans* (Berri et al., 2009). Across a wide range of environmental viscosities, from normal water to solid substrate, worms exhibit continuous, linear changes in kinematic (Berri et al., 2009) and muscle activity (Butler et al., 2015) parameters. This suggests a single underlying control scheme. In even more complex systems, using a gradient of environmental conditions may also be critical to a better understanding of the mechanisms of neuromuscular control (Gillis and Blob, 2001; Lutek et al., 2022a).

1.4 Investigating neuromuscular changes in an evolutionary context

Determining the neuromuscular control that facilitates behavioural change is essential to our understanding of behavioural plasticity. Behavioural plasticity is thought to be critical in evolutionary processes because it enables rapid responses to changing environmental conditions (West-Eberhard, 2003; Losos et al., 2004). One of the most dramatic evolutionary shifts in environment was from aquatic to terrestrial life history. Not only do these two environments pose dramatically different physiological challenges (reviewed in Wright and Turko, 2016), but the physical forces in these environments also impose unique requirements on the neuromuscular system to generate locomotion (reviewed in Lutek et al., 2022a). This means that, by necessity, even the first forays onto land would have required behavioural plasticity to allow the animal to leave and return to its aquatic habitat.

Understanding how this evolutionary transition occurred is a fundamental question in biology. However, the ability to test evolutionary hypotheses is limited in several ways. First and foremost, evolution occurs across generations. Thus, model organisms with a short generation time are essential to *in vivo* tests of the evolution of behaviour and neuromuscular control. Within-lifetime experiments can generate hypotheses about the nature of these changes but cannot explicitly test them. Second, evolution is directly contingent upon the set of selective pressures in a given system. This means that while we can generate proxies for hypothesized historical environments, or select natural environments where evolution is occurring, we cannot recreate the exact selective scenario that generated extant diversity. Paleontological records provide snapshots of the evolutionary path and, when combined with paleoclimate models, provide a reasonable estimate of the state space of selective pressures, but ultimately cannot provide any mechanistic certainty about the behavioural changes that occurred as aquatic animals first moved onto land.

Due to these limitations, we often investigate extant forms and systems with sufficient similarities to the extinct species and analogous evolutionary transitions of interest to generate

hypotheses about the mechanisms behind these historical transitions. The transition from water to land has been investigated from various perspectives including the physiological adaptations required for the transition, the morphological change across the fossil record, and the locomotor modifications that facilitate terrestrial locomotion (Ahlberg, 2019; Clack, 2009, 2012; Coates et al., 2008; Damsgaard et al., 2020; Dickson et al., 2021; Edwards, 1989; Gordon, 1998; Graham and Lee, 2004; MacIver and Finlay, 2021; Wright and Turko, 2016). A variety of extant fish and lower tetrapods have been proposed as models for different parts of the evolutionary transition including lungfish, mudskippers, salamanders, and amphibious fishes as a group (Aiello et al., 2014; Frolich and Biewener, 1992; Gordon, 1998; Graham and Lee, 2004; Horner and Jayne, 2014; Kawano and Blob, 2013; King et al., 2011; Pace and Gibb, 2014). Lungfish are of particular interest because of their close phylogenetic position to the common ancestor of fishes and tetrapods; however, the commonly available *Protopterus annectens* has filamentous pectoral and pelvic fins that preclude terrestrial walking and the lobe-finned *Neoceratodus forsteri* is an endangered species and thus not ideal for study (Brooks et al., 2019). Mudskippers are incredibly effective at moving on land; however, this is due to several morphological specializations that facilitate their unique crutching behaviour, which makes them not easily comparable to early tetrapods (Harris, 1960; Murdy, 1989; Pace and Gibb, 2009). Salamanders may be a good model for later stages of the fin-limb transition, but because of their derived morphology and well-developed limbs, they are not a useful model system for the first forays onto land (Kawano and Blob, 2013). Amphibious fish as a whole group can be used to investigate broad patterns of evolution in the water-to-land transition, but the staggering diversity of terrestrial locomotor styles across amphibious fish means testing mechanisms for moving from water to land first requires careful choice of a single species of interest.

Detailed study of a single extant species allows researchers to focus on a set of behavioural and neuromuscular changes that generate terrestrial locomotion and then compare these changes to

solutions in other species. This integrative approach allows the determination of common requirements for amphibious species moving on land (Gillis and Blob, 2001; Lutek et al., 2022a). Therefore, we can generate hypotheses about the sequence of behavioural, morphological, and physiological changes required for aquatic species to become amphibious by investigating the neuromuscular changes extant amphibious fish use to move on land.

1.5 Polypterus as a model of plasticity

Polypterus senegalus is a predominantly aquatic, extant fish that is phylogenetically closest to the common ancestor of actinopterygians and sarcopterygians. Like Elpistostegid fish, finned tetrapodomorphs of the late Devonian, *P. senegalus* has rhomboid scales, an elongate body, ventrolaterally-positioned fleshy fins, and paired lungs (Standen et al., 2014). While there is anecdotal evidence for emersion in *P. senegalus* (likely incidental due to drying of ephemeral pools and ponds in their native habitats in Africa), voluntary emersion has not been observed in the wild (Du et al., 2016). Despite this, *P. senegalus* respond to this apparently novel terrestrial environment on the acute time scale with effective tetrapod-like terrestrial walking on flat ground and an opportunistic, slithering-like behaviour across pebbles (Standen et al., 2014, 2016). This unique combination of early tetrapod-like morphology and behavioural plasticity in response to the terrestrial environment makes them an interesting study system for investigating the behavioural and neuromuscular requirements for an aquatic animal moving on land, as well as for generating hypotheses about how vertebrates took their first steps.

Previous work has investigated both the *activational* behavioural plasticity that facilitates walking, and the *developmental* plasticity in response to terrestrial acclimation and exercise in *P. senegalus*. *Activational* behavioural plasticity that generates terrestrial walking in *P. senegalus* is the result of muscle activation with discrete differences from that of swimming (Foster et al., 2018). In general, body and pectoral fin muscle activation intensity and duration are higher during walking than during

swimming. Additionally, the timing of pectoral fin adductor muscle activity (responsible for moving the pectoral fin towards the body during swimming) show characteristics of co-activation with the abductor muscle that would help stiffen the fin during walking. When kept on land for extended periods (eight months), *P. senegalus* become more effective walkers due to kinematic changes that reduce friction between the belly and ground (Standen et al., 2014). The bone and muscle morphology of *P. senegalus* is also plastic following terrestrial acclimation. The connection between the clavicle and cleithrum in the pectoral girdle becomes stronger, mirroring the trend in the stem tetrapods fossil record (Standen et al., 2014). Pectoral fin bones also become longer, wider, and more ossified (Du and Standen, 2017), and the pectoral fin muscles have a higher proportion of fast muscle fibers (Du and Standen, 2020). Similar, but smaller magnitude, changes in pectoral fin bone structure have also been identified following repeated terrestrial exercise of aquatically housed fish (Du and Standen, 2020). Together, this morphological and physiological *developmental* plasticity may help create more effective walking following time and/or exercise on land. *P. senegalus* is (a) plastic in response to novel environments and (b) comparable to late Devonian tetrapodomorphs, thus making it an ideal candidate for study. However, the neuromuscular mechanisms behind this behavioural plasticity have not been extensively investigated. My thesis aims to better elucidate how sensory feedback and mechanical constraint interact to generate behavioural plasticity in novel environments.

1.6 Aims and objectives

While the kinematics of aquatic and terrestrial locomotion have been characterized for a variety of amphibious fish, the muscle activity of only a small subset (including *P. senegalus*) have been characterized (reviewed in Lutek et al., 2022a), and fewer still have investigated the neuromuscular mechanisms that generate the observed changes. To this end, my thesis used *P. senegalus* to address four primary objectives: (1) to explore the extent of neuromuscular plasticity

across environmental gradients (viscosity and water depth), (2) to generate and test hypotheses about paramount signals for this neuromuscular plasticity, (3) to determine the neuromuscular underpinnings of locomotor transitions, and (4) to determine the neuromuscular control of developmental behavioural plasticity in novel environments.

Throughout my thesis I make use of kinematics and muscle activity in the framework described above to disentangle sensory feedback and mechanical constraint. I use environmental gradients of water viscosity (Chapter 2) and water depth (Chapter 3) to test how *P. senegalus* controls locomotion across resistive gradients and determine the nature of the swimming-walking transition in *P. senegalus*. I then perform the first experiments looking for a mesencephalic locomotor region in *P. senegalus* (Chapter 4) and test the role of this area for neuromuscular control in this species. Finally (Chapter 5), I determine the neuromuscular changes that help generate *developmental* behavioural plasticity in response to terrestrial acclimation and determine whether terrestrial exercise alone is sufficient to generate the previously observed behavioural changes. Together, these chapters test the overarching hypothesis that sensory feedback and mechanical constraint modulate the basic swimming neuromuscular pattern to generate locomotion across a variety of novel environments in *P. senegalus*.

1.7 Chapter summaries

1.7.1 Chapter 2: Increasing viscosity helps explain locomotor control in swimming P. senegalus

Chapter 2 is published in *Integrative Organismal Biology* (Lutek and Standen, 2021). The aim of this study was to use viscous water as a model system for determining how sensory feedback and mechanical constraint interact to generate effective motor control in *P. senegalus*. In contrast to a previously published sensory-deprived computer model (Tytell et al., 2010), our results demonstrated that increased muscle activity intensity generated exaggerated swimming kinematics as viscosity increased, resulting in consistent voluntary swimming speed across all viscosities. These

results suggest fish can adjust swimming motor control and kinematics using sensory feedback, while maintaining typical swimming coordination in high viscosity environments.

1.7.2 Chapter 3: Behaviour and muscle activity across the aquatic-terrestrial transition in Polypterus senegalus

Chapter 3 is under review in *Journal of Experimental Biology* (Lutek et al., 2022b; under review). This study focused on determining whether swimming and walking in *P. senegalus* are discrete locomotor modes or if they are opposite ends of a behavioural continuum that varies with the environmental gradient from aquatic to terrestrial. The results demonstrate that discrete changes in axial kinematics are driven by a continuum of red axial muscle activity, and that a discrete switch from in- to out-of-phase pectoral fin movements occurs when the pectoral fin first interacts with the substrate below. The findings suggest that both swimming and walking in *P. senegalus* are driven by swimming-like red axial muscle activity and similar pectoral fin muscle activity, and kinematics are constrained by environmental forces to have discrete changes. Together, this indicates that swimming and walking are not unique locomotor modes, but rather both are based on a common axial muscle activity pattern, and both axial and pectoral fin kinematic changes are the result of sensory feedback and physical constraints of the environment.

1.7.3 Chapter 4: Searching for the mesencephalic locomotor region in Polypterus senegalus

Chapter 4 describes a series of experiments aimed at localizing and testing the function of the mesencephalic locomotor region in *P. senegalus*. Despite limited success using electrical stimulation of the putative mesencephalic locomotor region to elicit regular locomotor kinematics, the results of a single successful preparation suggest that the mesencephalic locomotor region in *P. senegalus* controls the frequency of axial swimming undulations, but not locomotor transitions or pectoral fin movements. These preliminary findings highlight the potential value in successful identification of the mesencephalic locomotor region for clarifying the nature of locomotor control and locomotor transitions in *P. senegalus*.

1.7.4 Chapter 5: Plasticity of muscle activity and fiber type recruitment following terrestrial acclimation of an amphibious fish

The objective of chapter 5 was to determine the neuromuscular changes that generate *developmental* behavioural plasticity in terrestrial-reared *P. senegalus* and to understand the importance of static and dynamic loading for such changes. I demonstrate that both terrestrial acclimation and repeated terrestrial exercise of aquatically reared fish resulted in kinematic changes to the walking behaviour, although these changes were smaller in the latter. These kinematic changes were the result of subtle alterations in pectoral fin muscle activity timing and duration that helped fish reduce unnecessary friction between the pectoral fin and ventral aspect of the body with the substrate below. Thus, subtle changes in neuromuscular control permit more effective locomotor behaviour that could be a key step in the evolutionary transition from aquatic to terrestrial life history.

1.8 Figures

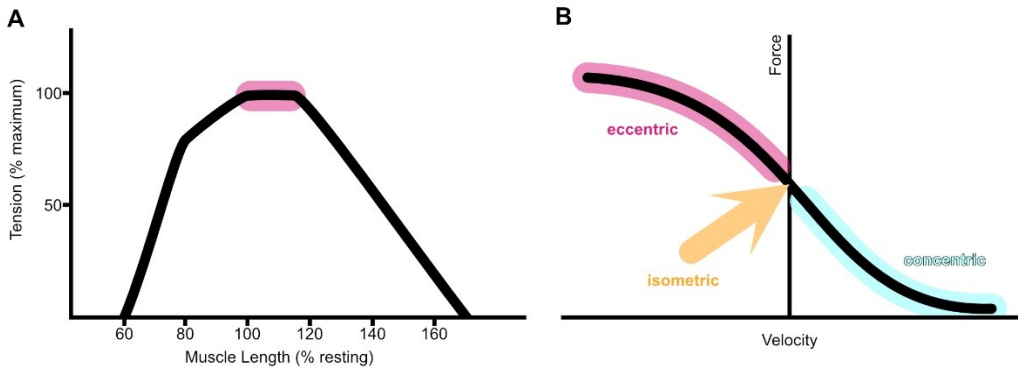

Figure 1.1. Muscle function changes with muscle length and contraction velocity. (A) Muscle force (tension) changes as muscle length changes. Around resting length, the muscle can produce maximal force; on either side (due to unideal overlap of actin and myosin heads) muscle force production decreases. (B) Muscle force production during eccentric (muscle lengthening) and concentric (muscle shortening) contractions.

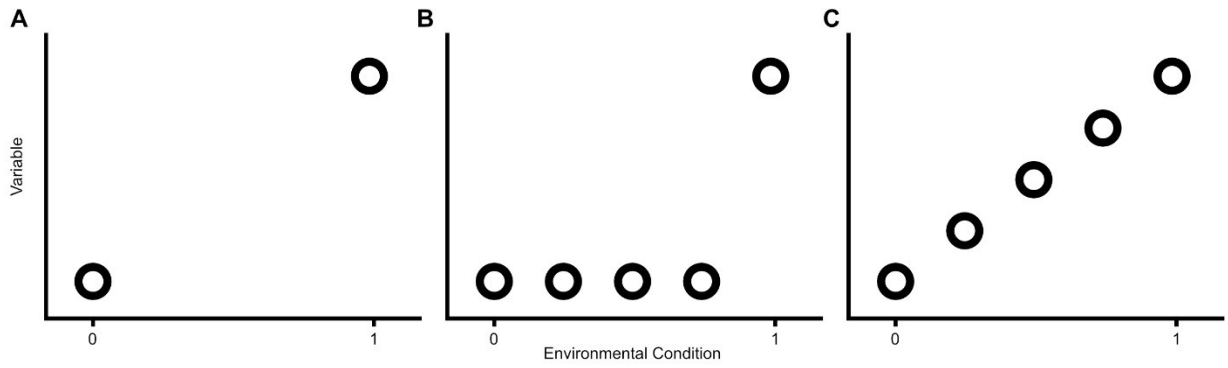

Figure 1.2. Testing for kinematic and muscle activity changes across environmental gradients elucidates discrete and continuous changes in motor control. (A) When a locomotor variable of interest is measured in two discrete environments, either a discrete change (B) or a continuous gradient (C) of neuromuscular and/or behavioural changes could be the cause of observed differences. Only by measuring these variables across an environmental continuum can the nature of the locomotor transition be determined.

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CHAPTER 2: INCREASING VISCOSITY HELPS EXPLAIN LOCOMOTOR CONTROL IN SWIMMING *POLYPTERUS SENEGALUS*

Chapter Notes

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Author contributions

K.L. and E.M.S. shared project conceptualization, experimental and methods design, code development, and writing of the manuscript. K.L. collected and analyzed the data.

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2.1 Abstract

Locomotion relies on the successful integration of sensory information to adjust brain commands and basic motor rhythms created by central pattern generators. It is not clearly understood how altering the sensory environment impacts control of locomotion. In an aquatic environment, mechanical sensory feedback to the animal can be readily altered by adjusting water viscosity. Computer modeling of fish swimming systems shows that, without sensory feedback, high viscosity systems dampen kinematic output despite similar motor control input. We recorded muscle activity and kinematics of six *Polypterus senegalus* in four different viscosities of water from 1 cP (normal water) to 40 cP. In high viscosity, *P. senegalus* exhibit increased body curvature, body wave speed, and body and pectoral fin frequency during swimming. These changes are the result of increased muscle activation intensity and maintain voluntary swimming speed. Unlike the sensory-deprived model, intact sensory feedback allows fish to adjust swimming motor control and kinematic output in high viscous water but maintain typical swimming coordination.

2.2 Introduction

Coordinated locomotion in vertebrates is the result of central pattern generators (CPGs), top-down signals (i.e., from higher brain centers such as cerebral cortex and including integrated sensory information), and local sensory feedback. Sensory feedback relays information from the environment to the central nervous system, altering motor output and ultimately fine-tuning locomotor performance. Higher order senses like vision relay environmental information to CPGs via the brain (e.g., cortex/pallium) (Bollmann, 2019; Severi et al., 2014). Local sensory feedback such as proprioception (e.g., muscle stretch receptors) and other more reflexive systems relay information directly to CPGs creating more immediate changes in motor output (Grillner et al., 1984; Viana di Prisco et al., 1990; Vinay et al., 1996). While each of these components is essential to adaptive, flexible locomotion (Kiehn, 2016; Garcia-Campmany et al., 2010; Goulding, 2009), how different sources of sensory feedback (either top-down or local) integrate and modulate locomotor behavior remains uncertain.

Fish use a variety of senses to tune their movements to their environment. Perturbations in flow demonstrate that fish use vision and lateral line sensing (both top-down sensory feedback systems) to control locomotion (Liao, 2007). Although little is known about proprioception in fishes, new research suggests that fish do rely on proprioceptive feedback in both the spinal cord and fins to fine-tune motor control (Aiello et al., 2020; Henderson et al., 2019). Further, since putative vertebral stretch receptor cells have been found in lamprey as well as a variety of basal vertebrates including snakes, salamanders, and elasmobranchs, it is likely that bony fishes also possess such cells (Massarelli et al., 2017). We can gain insight into how these sensory systems control locomotion by altering environmental conditions and watching how animal behavior changes. Highly viscous environments alter the mechanical forces experienced by an animal by increasing the boundary layer surrounding a swimming fish and decreasing the relative importance

of inertial forces (lowering Reynolds number [Re]). In other words, high viscosity systems are dampened, which may impact sensory feedback. Further, increased efforts are likely required to initiate and maintain motion in these environments, necessitating changes in motor control and affecting swim performance (Horner and Jayne, 2008; Tytell et al., 2010).

While most studies that have manipulated viscosity are aimed at questions outside of motor control (e.g., cold temperature metabolism, performance limits), they all indirectly suggest that sensory feedback is essential for adjusting to novel environmental mechanics (Danos, 2012; Danos and Lauder, 2012; Fuiman and Batty, 1997; Horner and Jayne, 2008; Hunt von Herbing and Keating, 2003; Johnson et al., 1998; McHenry and Lauder, 2005). Indeed, anguilliform swimming likely requires additional effort to maintain swim speed in a high viscosity environment, as is seen in lungfish swimming in high viscosity (Horner and Jayne, 2008). In addition, computer simulations of anguilliform swimming that lack sensory feedback, but maintain internal mechanics and muscle activation frequency, display dampened kinematics and decreased swim speed when environmental viscosity is increased (Tytell et al., 2010). These simulations also develop an increased phase lag between muscle activation and body curvature in a high viscosity environment. In contrast, a variety of intact fishes maintain voluntary swim speed as viscosity increases (steady swimming: Danos, 2012; Danos and Lauder, 2012; Fuiman and Batty, 1997; Horner and Jayne, 2008; Hunt von Herbing and Keating, 2003; Johnson et al., 1998; and unsteady swimming: McHenry and Lauder, 2005). To maintain speed in an increased viscous force environment, fish must actively change their muscle activation patterns, as seen in lungfish, which increase their muscle effort (rectified integrated area of the electromyography signal, RIA) and experience the predicted increase in phase lag between electromyography (EMG) onset and maximum body curvature when swimming through viscous media (Horner and Jayne, 2008). The signaling of this change in motor control is most likely due to one or many of the above mentioned sensory feedback systems. While muscle activation changes

kinematic output, the altered forces in the environment passively constrain kinematics, as is seen in the reduction of speed and amplitude of motion in sensory-deprived computer simulations (Tytell et al., 2010). In this study, we measure muscle activation patterns and resultant kinematic performance of the elongate bony fish, *Polypterus senegalus*, in a series of different viscous regimes. *P. senegalus* is the most basal actinopterygian. It has a predominantly aquatic life history, but also has the ability to locomote on land. Because of its phylogenetic position, and its ability to locomote amphibiously, *P. senegalus* has become an interesting evolutionary model and a considerable amount is known about how it changes motor control between its walking and swimming gaits. This study aims to understand *P. senegalus'* capacity to adjust to more subtle changes in environment, thus building a larger dataset with which to understand how sensory feedback and environmental forces impact motor control. By leaving sensory systems intact and manipulating the environment, we discuss how sensory feedback systems, combined with passive mechanical constraint, may be involved in motor control in intact animals.

2.3 Materials and Methods

2.3.1 Animals

Polypterus senegalus were acquired from the pet trade (AQUAlity Tropical Fish Wholesale Inc., Mississauga, ON, Canada). Fish were kept in individual recirculating (10% water change each week) flow-through tanks on a 12/12 light cycle at 25–26°C. Six fish (total length: 136.33 ± 1.32 mm; mass: 14.55 ± 0.54 g) were used for kinematic and EMG experiments. All fish swam in 1 cP (normal water viscosity) and 40 cP water. Three of these fish also swam in 5cP and 10cP water. Fish numbers were chosen based on variation seen in previous experiments. All experiments were performed according to University of Ottawa Animal Care Protocol BL-2069.

2.3.2 High-speed videography

Water viscosity was altered by the addition of methyl cellulose (400 cP; M0262, Sigma-Aldrich) and measured before each experiment using either a S1 or S2 Shell Cup® (Norcross Corporation, Newton, MA). Note that at the concentration necessary to achieve a viscosity of 40 cP (<1%w/w), we would expect the solution to exhibit Newtonian behavior (Herráez-Domínguez et al., 2005). Fish swam in a standing water tank (5.4 cm × 80 cm) and were filmed from below by a Photron Fastcam Mini UX (Photron USA Inc., San Diego, CA) at 250 frames per second. The height of the water for all trials was 5 cm. All experiments were run with room temperature water (23–24°C). Fish were in each condition for no more than 10 min to minimize stress and were given free access to the surface to breathe using their lungs, as desired. The order that the fish were exposed to each viscosity was randomized to minimize any order effects of the different conditions. Videos were analyzed only if the fish completed three or more steady locomotor cycles (tail fin beats) in a row (Movies S2.1 and S2.2). A total of 5-10 cycles were analyzed for each fish in each condition. The nose and tip of the caudal fin was digitized using DLT Data Viewer 6 (Hedrick, 2008). Videos were binarized and fish midlines (from nose to tip of caudal fin) were automatically tracked using custom Matlab code. Frame numbers and x-y coordinates of the pectoral fin lobes when the fin began adduction and abduction were identified in FIJI (Schindelin et al., 2012).

2.3.3 Kinematics analysis

The following variables were calculated for each video sequence: swim speed (BL s^{-1}; BL, body length), maximum body curvature (calculated over 100 equal length segments along the entire length of the fish; BL^{-1}), body wave speed (traveling wave of maximum body curvature passing along three sections of the fish: 35–55% BL, 55–75% BL, and 75–95% BL; BL s^{-1}), body wave frequency (cycles s^{-1}), fin frequency (defined by the start of pectoral fin adduction; cycles s^{-1}), fin angle at start of adduction (supplementary angle to the angle between the tip of the nose, back of skull and tip of

the pectoral fin lobe; radians; Fig. 2.1), fin angle at the start of abduction (radians; Fig. 1), and Re. Values for body curvature and body frequency were calculated at each position where they could be reliably detected. Therefore, body curvature was not calculated for the head or tail, and body frequency was not calculated for the head or the two most anterior electrode positions. All variables were calculated using custom code in Matlab (version R2018/2019, The MathWorks, Natick, MA).

2.3.4 Electromyography

Prior to surgery, fish were lightly anesthetized in 200mg L^{-1} buffered tricaine methanesulfonate (MS222, Syndel Laboratories Ltd., BC, Canada). During surgery, fish were kept moist and anesthetized with holding tank water and anesthetic. Two-pronged electrodes were fashioned out of 0.051 mm insulated bi-filament stainless steel wire (California Fine Wire Company, Grover Beach, CA, USA) and implanted in the fish with 27 gauge needles (Sigma-Aldrich). Electrodes were implanted in the red muscle zone <1mm deep at five locations just above the lateral line down the left side of the body (just behind the pectoral fin base, 20.5 ± 0.008% BL; just anterior to the anal fin, 69.2 ± 0.013% BL; and three locations evenly spaced in between: 33.8 ± 0.015% BL, 47.6 ± 0.013% BL, and 59.4 ± 0.012% BL) (Fig. 2.1). To compare left- and right-side body muscle timing, two electrodes were placed on the body's right side at the second and fourth positions (34.6 ± 0.013% BL and 61.2 ± 0.012% BL, respectively) (Fig. 2.1). Finally, one electrode was placed in the right pectoral fin adductor muscle (Fig. 2.1). Electrode wires were secured to the dorsal finlets with suture to reduce strain and the fish was allowed to recover in tank water. EMG signals were recorded using a Grass P511 AC Amplifier (Grass Instrument Company, West Warwick, RI, USA), fed through a Powerlab 16/35 digital-to-analog converter (AD Instruments, Colorado Springs, CO, USA) and recorded in Lab Chart 8 (AD Instruments). Signals were recorded at 10 kHz with a 60 Hz notch filter to eliminate ambient electrical noise and a 40–4000 Hz band pass filter to eliminate movement artifacts, and analyzed with custom Matlab code. Video and EMG recordings were

synchronized using an external trigger. Following euthanasia (MS222 417 mg L^{-1} in housing water), mass and length were recorded and electrode locations were confirmed by dissection.

To facilitate comparison of muscle intensity across individuals, we found the maximum value EMG recorded over all trials, for each electrode (EMG_{ExpMax}). Rectified integrated area under the EMG curve (RIA) is reported as a percentage of theoretical maximum RIA for each muscle burst (theoretical maximum RIA=EMG_{ExpMax} × burst duration). Muscle burst start and stop times were manually identified from the EMG trace and used to calculate burst duration in % tail beat cycle duration (EMG duty factor) and RIA(a measure of how hard a muscle is working, described above). Timing of body muscle onset relative to the timing of maximum body curvature was calculated as a percentage of the tail beat cycle duration (EMG onset-curvature phase lag). Note that most EMG variables are presented only for the left-side electrodes. EMG data for right-side electrodes are used only to test for differences in timing of contralateral muscle contraction.

2.3.5 Statistical analysis

Linear statistics and graphing were carried out in R 3.3.1 (R Team Core, 2018) using the tidyverse package (Wickham et al., 2019). We calculated trial averages and used these as our observations for all models (each fish performed two to three trials in each condition). Linear mixed-effects models were created in nlme (Pinheiro et al., 2018) with viscosity, position, and the interaction of these two variables (when applicable, based on AIC model comparisons) as fixed effects and individual as a random effect. For those variables that were measured at multiple body positions, the random effects were modeled as body point nested in individual to account for potential differences in variation across body positions. Given the number of individuals in our dataset, we have chosen the most conservative estimation of degrees of freedom for this type of model as defined by Pinheiro and Bates (2000). Results for all linear mixed-effects models are reported as ANOVA-type tables. When necessary, corrections were made for unequal variance of

the residuals across body positions using the constant variance function (varIdent) in nlme (Pinheiro et al., 2018). Pseudo-R-squared values were calculated for each model using MuMIn (Barton, 2018). When necessary, post-hoc multiple comparisons were performed across treatments within a position and across positions within a treatment using the estimated marginal means of each model (Length, 2019). P-values were Bonferroni-corrected based upon the total number of pairwise comparisons performed for each dependent variable. Graphs for linear variables were created using ggplot2 (Wickam, 2016). Note that Re values and their estimated marginal means are reported non-transformed for clarity but, to account for non-normal residuals, were log-transformed for statistical analysis.

2.4 Results

2.4.1 Electrode effects

A paired comparison of swimming in each treatment before and after electrode placement was conducted to quantify the effect of EMG electrodes on kinematic behavior. Following electrode implantation (and accounting for speed as a covariate in the model), pectoral fin range of motion remained constant; however, fin angles at the start of both adduction and abduction were 20° larger and pectoral fin frequency was 0.7 cycle s^{-1} faster. All other kinematic variables collected showed no difference following electrode implantation. Due to the lack of differences across most variables and to pair kinematics with muscle data, we have included only post-EMG surgery trials in this analysis. Individuals were swum across all viscosity treatments to allow paired comparisons.

2.4.2 Kinematic and muscle activity changes along the body

In order to understand the anterior-posterior changes in swimming kinematics and motor control, we examined the trends in our variables along the body of the fish. Body curvature increased toward the fish's tail in all treatments (Tables 2.1 and 2.2; Fig. 2.2A). This increase is statistically significant at position 5 in all treatments, and also significant at position 4 in 40 cP. In

each treat- ment, body frequency was consistent across body points (Tables 2.1 and 2.2; Fig. 2.2B). The posterior body (75–95% BL) had a significantly higher wave speed than more anterior body positions (35–55% and 55–75% BL) (except in 5 cP at 35–55% BL; Tables 2.1 and 2.2; Fig. 2.2C). Body EMG duty factor and body muscle RIA did not vary across electrode positions 3–5 in any treatment (Table 2.1; Fig. 2.3). EMG onset-curvature phase lag was significantly affected by position according to ANOVA- type results (Table 2.1), but this difference was not large enough to remain following correction for multiple comparisons (Table 2.2; Fig. 2.4).

2.4.3 Kinematic changes across viscosities

Since the outward kinematics are a combination of motor control and environmental constraint, we report changes in kinematics to understand how successful locomotion in each viscosity was achieved. There was no significant change in swimming speed across all viscosities (Tables 2.1 and 2.3), despite a significant decrease in Re (Tables 2.1 and 2.3). Fish displayed significantly larger body curvature in 40 cP than in 1 cP for positions 3-5 (Tables 2.1 and 2.3; Fig. 2.2A). There was a significant positive relationship between body frequency and viscosity at all body points at or posterior to position 2 (Tables 2.1 and 2.3; Fig. 2.2B). Wave speed was significantly higher in 40 cP than in 1 cP along the posterior of the fish (Tables 2.1 and 2.3; Fig. 2.2C). Increased viscosity resulted in a higher pectoral fin frequency (Tables 2.1 and 2.3). There was no change in fin adduction or abduction angle across viscosities (Tables 2.1 and 2.3).

2.4.4 Electromyography changes across viscosities

EMG magnitude and timing were quantified to determine whether the motor pattern changed due to increased viscosity. Quantitative data are only presented for those electrode positions where there was enough data in all viscosities for complete linear models, as described above. Since burst presence at positions 1 and 2 during swimming in 1 cP and 5 cP is inconsistent (present in <10% of the tail beat cycles analyzed in each condition), EMG data were analyzed for

only positions 3 through 5 (Fig. 2.1). Body EMG duty factor did not change across viscosities (Tables 2.1 and 2.3; Fig. 2.3A). Body RIA increased significantly in 10 and 40 cP for all applicable body electrode positions, while pectoral fin RIA increased significantly in 40 cP (Tables 2.1 and 2.3; Fig. 2.3B). EMG onset-curvature phase lag was significantly affected by viscosity in the ANOVA-type results (Table 2.1), but all differences became nonsignificant after correction for multiple comparisons (Table 2.3; Fig. 2.4). Note that in support of a unilateral, alternate pattern of body muscle activity that typifies swimming (in other words no presence of co-contraction in a more viscous system), neither left- nor right-side electrodes show any indication of a change in body EMG duty factor (consistently <50% cycle duration; linear mixed-effects model, $P > 0.05$) or EMG onset timing (as a percentage of tail beat cycle duration; linear mixed-effects model, $P > 0.05$) across viscosities.

2.5 Discussion

Computer simulations of swimming that maintain motor control patterns but increase water viscosity show that swimming speed, body amplitude, and body frequency decrease in the absence of sensory feedback (Tytell et al., 2010). In short, an increase in viscosity dampens the system. If living systems were without sensory feedback, we would expect to see the same dampening of swimming kinematics. In contrast, *P. senegalus* maintain their swimming speed even when water is 40x more viscous than normal and do so by increasing their body and pectoral fin muscle effort (RIA). Our results are in line with the few previous studies that have shown adult fish maintain swimming speed in viscous water (Danos and Lauder, 2012; Horner and Jayne, 2008; Johnson et al., 1998), and accord with clear evidence that some fishes have a preferred swimming speed related to energy consumption (Beamish, 1978). The changes in muscle activity we report for *P. senegalus* are similar to those in lungfish (the only other investigation of fish muscle activity in viscous water), suggesting that these two phylogenetically distinct animals respond to this novel environment in

similar ways. The maintenance of swimming speed in living fish suggests sensory feedback systems fine-tune motor control to address the constraints of this novel environment.

2.5.1 Vision

Fish use vision to facilitate steady swimming in both flowing and still water (reviewed in Liao, 2007). Visual flow allows fish to perceive swim speed relative to their surroundings and thus helps tune kinematics and muscle activity to achieve a preferred swimming speed (Ahrens et al., 2012). Indeed, migrating salmonids appear to maintain their ground speed at roughly 0.5 BL s^{-1} regardless of the river flow velocity they encounter (Beauchamp et al., 1989). Increasing viscosity does not remove visual cues and *P. senegalus* maintained voluntary swimming speed despite the increase in mechanical constraint, suggesting that despite poor visual acuity (Znotinas and Standen, 2019), visual feedback could be used by *P. senegalus* to determine preferred swim speed.

2.5.2 Lateral line

How other sensory feedback systems modify swimming performance in high viscosity requires closer examination of the body kinematics and muscle timing. In high viscosity, *P. senegalus* increase their body curvature, the frequency of their pectoral fin and body motions, and the speed of the body wave as it travels posteriorly. Each of these kinematic variables has the capacity to impact the environmental signals received by mechanical sensory feedback systems. For example, lateral line cells detect flow velocity and acceleration along the fish's length that can be used to tune swimming movements (Bleckmann and Zelick, 2009; Coombs et al., 2001; Engelmann et al., 2000; McHenry et al., 2008). The velocity and thickness of the boundary layer around the fish directly affect the signal received by superficial neuromasts (i.e., they are speed sensitive), and indirectly affect canal neuromasts (i.e., they are acceleration sensitive) (McHenry et al., 2008; Rapo et al., 2009; van Netten, 2006; Windsor and McHenry, 2009). In a more viscous (lower Re) environment, the boundary layer is expected to be larger and thus more similar to that of a slowly swimming fish (Windsor and

McHenry, 2009). An increase in body motion (both increased curvature and wave speed) in high viscosity would cause an increase in local flow speed along the body, and should decrease the boundary layer surrounding the fish, increasing feedback to the lateral line cells. High-frequency body undulations may have the same effect, increasing the response of lateral line cells (McHenry et al., 2008; van Netten, 2006). While there are mixed reports about the role of efferent neurons working to filter self-generated hydrodynamic signals during steady swimming (e.g., evidence for such activity [Roberts and Russell, 1972] and against [Mensinger et al., 2019]), it does appear that the lateral line of fish is responsive to normal swimming body movements. These signals may either be interpreted as a source of information (e.g., when body movements are unexpected) or as a source of noise (e.g., when body movements are expected), depending on the situation (Montgomery et al., 2009). Both afferent and efferent lateral line signaling evidently require further investigation, but they present potentially important sources of information for fish swimming in an increased viscosity environment. The role of lateral line feedback in the observed responses could be tested by swimming *P. senegalus* in viscous water following a block of their lateral line cells. If lateral line sensory feedback is important, as we propose here, we would expect fish with a blocked lateral line in a high viscosity environment to have no change in muscle activity, and therefore demonstrate a dampened kinematic output.

2.5.3 Mechanical constraint

Independent of sensory feedback, mechanical constraint most likely contributes to kinematic differences between water and high viscosity and is best represented by the phase lag between muscle activation (i.e., EMG onset) and resultant body motion. Based on a model of undulatory swimming, an increase in this phase lag can be a direct consequence of interactions between relatively high fluid forces and relatively weak body forces (e.g., muscle force, spring forces), as is the case in high viscosity environments (Tytell et al., 2010). Presumably, the increased phase lag results

because (for a given muscle force) the time interval between initiating muscle activity and resultant kinematic output should be inversely related with the resistive forces caused by the viscosity of the environment. Thus, the environment constrains the timing of kinematic output. Although not statistically significant (Table 2.1; Fig. 2.4), this constraining effect is present, as despite increases in muscle effort (which might balance environmental resistance), EMG onset-curvature phase lag tends to in- crease in *P. senegalus* as viscosity increases. A significant increase in phase lag is achieved in lungfish when viscosity reaches 100 times that of water (Horner and Jayne, 2008). Since our highest viscosity is only 40 times more viscous than normal water, it is possible that we would see a statistically significant magnitude shift in *P. senegalus* at higher viscosities. Interestingly, our fish were swimming at similar Re in 40 cP water to lungfish moving in ~100 cP water. Thus, the dynamics of these systems are similar, suggesting that something other than simply mechanical constraint is influencing the observed phase lag.

Whether the change in EMG onset-curvature phase lag observed in both lungfish and *P. senegalus* is solely due to passive mechanical constraint, is unclear from intact animal data; the observed lag could be contributed to active shifts in body stiffness as well. Regardless, the functional consequences of this shift remain the same. Shifting EMG onset earlier in the curvature cycle (assuming consistent EMG duty factor as in our dataset) changes the amount of positive work done by the muscles. While body curvature is not a perfect proxy for muscle length change timing, shifting EMG onset earlier relative to maximum curvature makes it more likely that muscles are active during lengthening. If present, negative work would help stiffen the body of the fish and facilitate power transfer to the posterior of the body while swimming through a high resistance fluid. The larger lag in lungfish than in *P. senegalus* in similar Re environments may be due to the dermis and scales of *P. senegalus* stiffening the fish more than the skin of lungfish (Long et al., 1996). If so, *P. senegalus* would have less need to actively stiffen the body to permit effective transfer of power to the

caudal fin for swimming and so would show a smaller increase in phase lag between EMG onset and max body curvature.

2.5.4 Similarities between high viscosity and high-speed swimming

A lag between EMG onset and max curvature is also observed during high-speed swimming in a variety of fishes (e.g., Coughlin, 2000; Jayne and Lauder, 1995a), suggesting high viscosity swimming is coopting a natural swimming control pattern. Generally, when fish swim faster, wave speed, tail beat frequency, pectoral fin beat frequency (when present), and muscle activity increase (to facilitate the more powerful movements) while maintaining a unilateral, alternate pattern of muscle activity along the body (e.g., Coughlin, 2000; Jayne and Lauder, 1995a, 1995b). As speed increases, absolute body EMG burst duration decreases to maintain duty factor with an increased tailbeat frequency (Shadwick et al., 1998). Interestingly, as water viscosity increased, *P. senegalus* showed these same changes in kinematics and muscle activity but without an accompanying change in speed (Tables 2.1 and 2.3; Figs. 2.1-2.3). Note that because the observed increase in wave speed occurs without a concurrent increase in swimming speed (i.e., slip or propeller efficiency decreases), it suggests that swimming in viscous water is less efficient than swimming in normal water.

Elongate fish including eels, needlefish, and gar (Liao 2002; Long et al. 1996; Tytell 2004) have shown a positive relationship between swim speed and body amplitude. Since *P. senegalus* are also elongate and increase body curvature (and amplitude) at increased viscosities, this may also suggest *P. senegalus* coopt changes in motor control typically used to increase swim speed. However, whether a positive relationship between body amplitude and swim speed is observed in *P. senegalus* remains to be determined. *Polypterus senegalus* also increase the frequency of pectoral fin cycles in high viscosity, but keep angle at the start of adduction and abduction constant, a change typical of Labriform propulsion as speed increases (e.g., Drucker and Jensen, 1996; Walker and Westneat, 1997). *Polypterus senegalus* may move the pectoral fin in the same arc because at these muscle lengths,

despite changes in fin cycle frequency, both work and power output are maximal (e.g., seahorse dorsal fin muscle; Ashley-Ross, 2002). Deviating from this range of motion would therefore diminish the ability to overcome the increased resistance of a high viscosity environment or to increase swimming speed. Thus, the changes in kinematics and muscle activity of *P. senegalus* while swimming in viscous water mirror changes observed when fish increase swimming speed.

2.5.5 Possible local sensory feedback mechanisms

Increasing viscosity increases the mechanical dampening of the environment acting on sensory systems. Most likely, sensory information from local (proprioception) and higher order (vision and lateral line) systems work in concert to achieve the exaggeration of body and fin swimming motions we see in *P. senegalus* experiencing higher viscosity. While there is still little direct evidence for proprioceptive senses in bony fish (but see Aiello et al., 2020; Henderson et al., 2019), several studies have demonstrated stretch-receptive cells in the spinal cord of lamprey and dogfish (Grillner and Wallén, 1982; Grillner et al., 1981a, 1981b, 1984). These cells change firing frequency in response to local bending angle and velocity (Massarelli et al., 2017). Both stretch receptors and proprioceptors input directly into local circuits (and stretch receptors also provide feedback directly to the reticulospinal neurons), facilitating rapid adjustment of locomotor movements to changes in environment (Hsu et al., 2016). In an environment with increased mechanical resistance, like that caused by an increase in viscosity, a given intensity of muscle activity would result in less local bending (Tytell et al., 2010). The observed increased muscle activity in *P. senegalus* swimming in high viscosity water may have been the result of feedback from stretch receptors or proprioceptors signaling the need for increased local bending. The fact that increased muscle activity resulted in an increase and not just the maintenance of local bending suggests that if such local sensory feedback exists in *P. senegalus* it was either overcompensating or working in combination with other sensory feedback systems to augment movement. Indeed, whether stretch-receptive or bending-sensitive

proprioceptive cells are present in *P. senegalus* remains to be seen and confirmation would require direct cell recordings at different levels of bending, as has been done in dogfish and lamprey (Grillner and Wallén, 1982; Grillner et al., 1981a, 1981b, 1984).

2.6 Conclusion

Mathematical models suggest that without sensory feedback, increased mechanical constraint at high viscosity will result in dampened swimming kinematics. The results we present here suggest that in living animals, sensory-driven changes actually increase swimming kinematics in a high viscosity environment. These results suggest that manipulating viscosity in combination with alteration to different sensory feedback systems could shed light on key sensory inputs that impact motor control in novel environments.

2.7 Figures & Tables

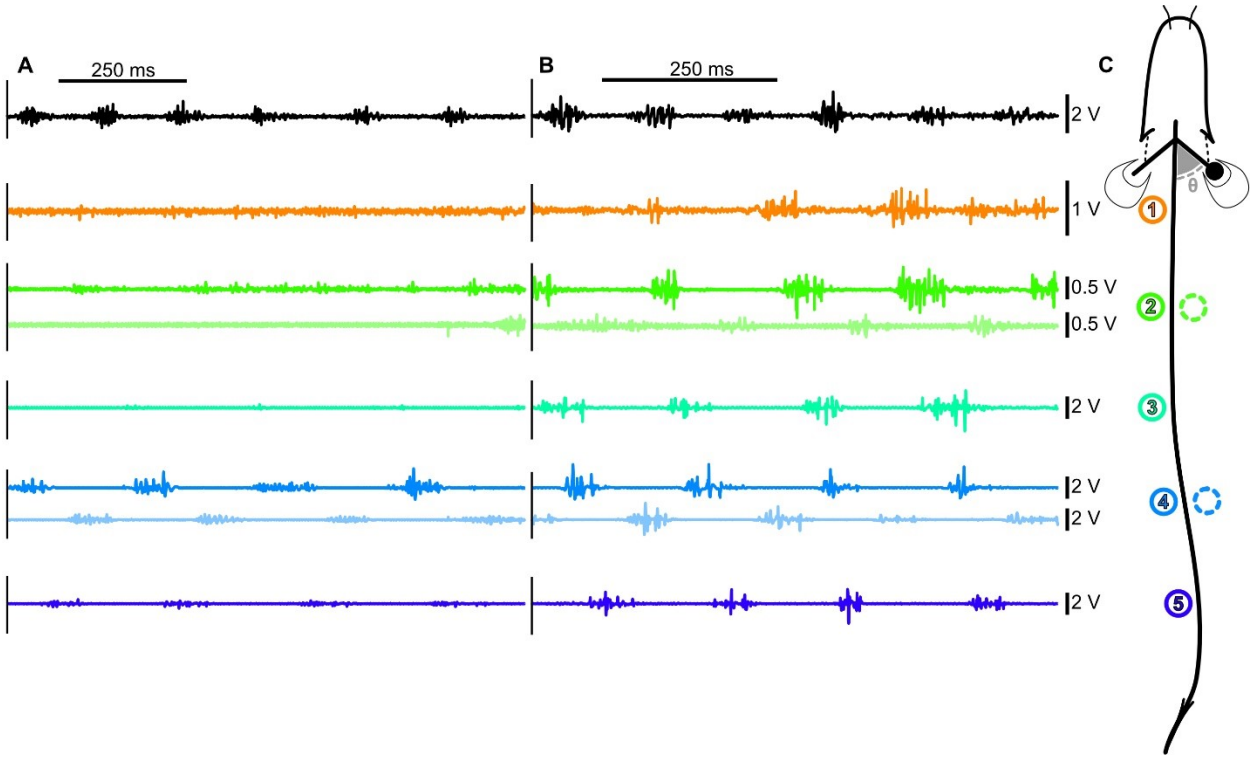

Figure 2.1. Representative traces of muscle activity, electrode position and pectoral fin angle. Muscle activity in 1 cP water (A) and 40 cP (B) for the pectoral fin and left-side body electrodes. Electrode position and pectoral fin angle calculation for all fish (C). Five electrodes were implanted on the left side of the body spaced equally between the posterior edge of the pectoral fin and the anterior edge of the anal fin. Two electrodes were implanted on the right side to match body positions 2 and 4 on the left side. One electrode was implanted in the adductor muscle of the right fin. Fin angle was calculated as the supplementary angle to that made by the head, 20% body length (BL; approximate position of the pectoral girdle) and the middle of the tip of the pectoral fin lobe (denoted θ in panel C). Pectoral fin adductor (black), electrode 1 on the left side (20.5 ± 0.008% BL; orange), electrode 2 on the left side (33.8 ± 0.015% BL; green), electrode 3 on the left side (47.6 ± 0.013% BL; cyan), electrode 4 on the left side (59.4 ± 0.012% BL; light blue), and electrode 5 on the left side (69.2 ± 0.013% BL; dark blue). Traces for electrodes on the right side are shown directly under the matching left-side trace at 50% transparency as indicated by the color matching positions in panel C.

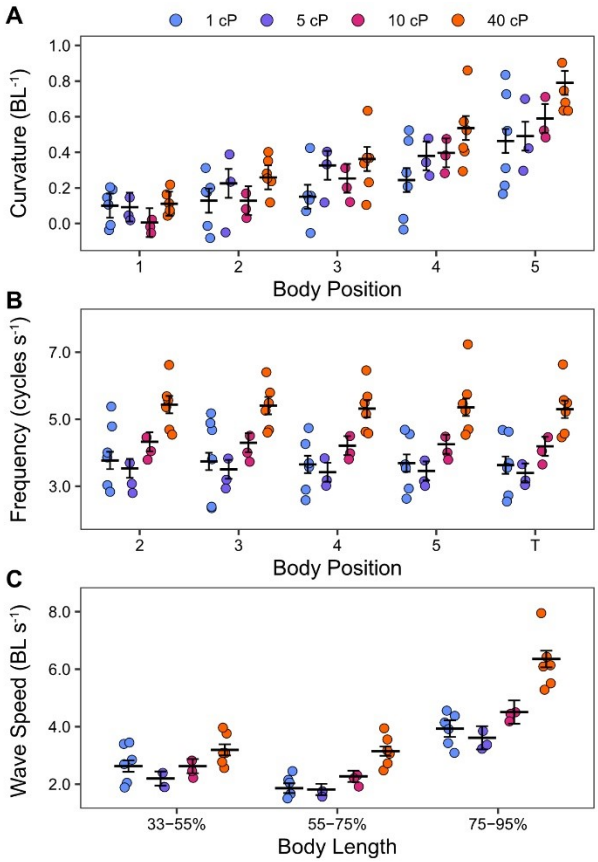

Figure 2.2. Kinematics of *P. senegalus* swimming in viscous water. Data from the five electrode positions on the left side of the body (as in Fig. 1C) and the tail are shown along the x-axis in panels A and B. In panel C, wave speed is shown across three portions of the body of the fish. Curvature, wave speed, and wave frequency increase significantly as viscosity increases at all positions measured ($P < 0.05$).

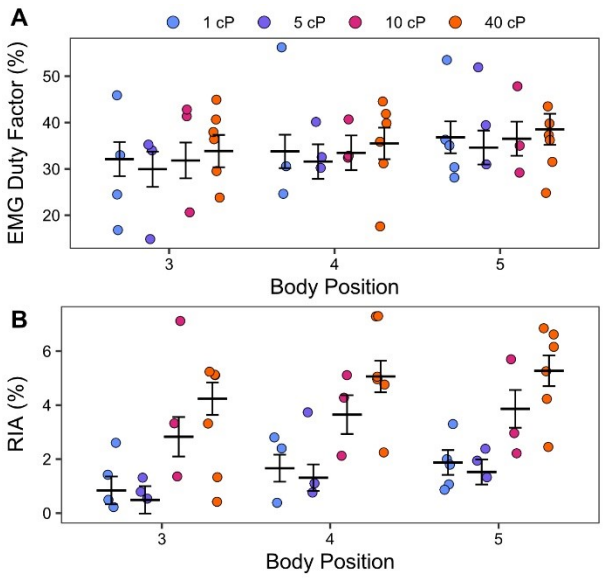

Figure 2.3. Muscle activity of *P. senegalus* swimming in viscous water. Data from the three most posterior electrode positions (3-5; as in Fig. 1C) on the left side of the body are shown along the x-axis. Burst duration (presented in % cycle duration) remains constant as viscosity increases, whereas RIA (presented in % theoretical maximum RIA) increases significantly ($P < 0.05$).

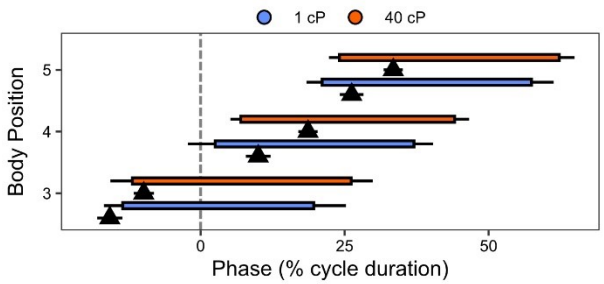

Figure 2.4. Timing of EMG onset relative to maximum curvature for 1 cP and 40 cP. Mean EMG onset timing is the start of the horizontal bar; mean EMG offset timing is the end of the horizontal bar (standard error is the black line at each end of the muscle activity). Triangle underneath each bar indicates the mean timing of maximum curvature Start of cycle (0%) is the tail starting to move to the right. Middle of (mean = triangle apex; standard error = horizontal bar on triangle). the cycle (50%) is the tail starting to move to the left. Note that phase lag increases toward the posterior of the fish.

Table 2.1. Summary of F values for linear mixed effects models fitted to kinematic variables.

Variable	R^2m [R^2c]	Viscosity	Position	Viscosity X Position
Speed (BL/s)	0.181 [0.244]	2.74 (3,29)	n/a	n/a
Re	0.960 [0.962]	305.24* (3, 29)	n/a	n/a
Body Curvature (BL^{-1})	0.530 [0.770]	0.84 (3, 143)	9.52* (4,20)	2.57* (12,143)
Wavespeed (BL/s)	0.837 [0.885]	5.87* (3, 81)	30.87* (2, 10)	4.14* (6, 81)
Body Frequency (cycles s^{-1})	0.435 [0.634]	70.54* (3, 157)	0.24 (4, 20)	-
Fin Frequency (cycles s^{-1})	0.416 [0.605]	12.35* (3, 29)	n/a	n/a
Fin Angle – Start Adduction (rad)	0.161[0.275]	2.72 (3, 29)	n/a	n/a
Fin Angle – Start Abduction (rad)	0.043[0.710]	1.47 (3,29)	n/a	n/a
Body EMG Duty Factor (%)	0.054 [0.516]	0.97 (3, 74)	0.82 (2, 10)	-
Body EMG RIA (%)	0.647 [0.857]	30.34* (3, 74)	1.56 (2, 10)	-
Pectoral Fin EMG Duty Factor (%)	0.011[0.667]	0.34 (3, 28)	n/a	n/a
Pectoral Fin EMG RIA (%)	0.217[0.357]	3.99* (3, 28)	n/a	n/a
EMG Onset-Curvature Phase Lag (%)	0.175 [0.380]	3.17* (3, 73)	7.31* (2,10)	-

R^2m is for the fixed effects in the model. R^2c is for the whole model. The F-value and degrees of freedom (numerator, denominator; in parentheses after each F-value) are presented for the fixed effects viscosity, position, and the interaction of viscosity and position. Asterisk denotes $P < 0.05$. Dash indicates a term not included in the model (either due to being used to calculate the dependent variable or based on AIC criterion best fit model). "n/a" is placed in cells where there is only one position possible. Position is the position on the body of the fish and was included in models only for those variables where data were quantified at multiple positions.

Table 2.2. Connecting letter reports based on Bonferroni-corrected p-values for kinematic variables across body position, within a given condition.

Variable	Viscosity	Position						
		H	1	2	3	4	5	T
Body Curvature (BL^{-1})	1 cP	-	a	a	a	ab	b	-
	5 cP	-	a	ab	ab	ab	b	-
	10 cP	-	a	ab	abc	bc	c	-
	40 cP	-	a	a	ab	bc	c	-
EMG Onset-Curvature Phase Lag (%)	1 cP	-	-	-	a	a	a	-
	5 cP	-	-	-	a	a	a	-
	10 cP	-	-	-	a	a	a	-
	40 cP	-	-	-	a	a	a	-
		35-55			55-75		75-95	
Wavespeed (BL/s)	1 cP	a			a		b	
	5 cP	ab			a		b	
	10 cP	a			a		b	
	40 cP	a			a		b	

Report is given only for the left-side electrode positions; values for right-side electrode positions 2 and 4 are comparable to left-side electrodes 2 and 4. Estimated marginal means and their standard error can be found in Table 3. Position is the position on the body of the fish and was included in models only for those variables where data were quantified at multiple positions.

Table 2.3. Connecting letter reports for kinematic and EMG variables across viscosity, within a given condition.

Variable	Position	Viscosity			
		1 cP	5 cP	10 cP	40 cP
Speed (BL/s)	-	0.65[0.05]^{a}	0.42[0.07]^{a}	0.51[0.07]^{a}	0.59[0.05]^{a}
Re	-	11384[1.10]^{a}	1620[1.09]^{b}	953[1.10]^{c}	257[1.10]^{d}
Body Curvature (BL^{-1})	1	0.100[0.068]^{a}	0.092[0.081]^{a}	0.005[0.081]^{a}	0.111[0.067]^{a}
	2	0.129[0.068]^{a}	0.225[0.081]^{a}	0.128[0.081]^{a}	0.259[0.067]^{a}
	3	0.151[0.067]^{a}	0.327[0.081]^{ab}	0.254[0.081]^{ab}	0.363[0.067]^{b}
	4	0.244[0.067]^{a}	0.380[0.081]^{ab}	0.397[0.081]^{ab}	0.537[0.067]^{b}
	5	0.464[0.067]^{a}	0.491[0.081]^{a}	0.590[0.081]^{ab}	0.790[0.067]^{b}
Wavespeed ($BL\ s^{-1}$)	35-55%	2.63[0.20]^{ab}	2.19[0.25]^{a}	2.63[0.25]^{ab}	3.19[0.19]^{b}
	55-75%	1.86[0.17]^{a}	1.82[0.20]^{a}	2.27[0.20]^{a}	3.15[0.16]^{b}
	75-95%	3.93[0.29]^{a}	3.61[0.41]^{a}	4.51[0.41]^{a}	6.35[0.29]^{b}
Frequency ($cycles\ s^{-1}$)	2	3.77[0.26]^{ab}	3.53[0.28]^{a}	4.33[0.28]^{b}	5.43[0.26]^{c}
	3	3.74[0.26]^{ab}	3.51[0.28]^{a}	4.30[0.28]^{b}	5.40[0.26]^{c}
	4	3.65[0.26]^{ab}	3.42[0.28]^{a}	4.21[0.28]^{b}	5.32[0.26]^{c}
	5	3.69[0.26]^{ab}	3.46[0.28]^{a}	4.25[0.28]^{b}	5.36[0.26]^{c}
	T	3.63[0.26]^{ab}	3.40[0.28]^{a}	4.19[0.28]^{b}	5.30[0.26]^{c}
	F	5.71[0.26]^{a}	5.46[0.34]^{a}	6.11[0.34]^{a}	7.07[0.26]^{b}
Fin Angle – Start Adduction (rad)	-	1.48[0.01]	1.48[0.02]	1.45[0.02]	1.44[0.01]
Fin Angle – Start Abduction (rad)	-	1.25[0.04]	1.27[0.04]	1.30[0.04]	1.24[0.04]
EMG Duty Factor (%)	3	32.1[3.7]	29.9[3.81]	31.8[3.9]	33.9[3.5]
	4	33.8[3.6]	31.6[3.7]	33.5[3.7]	35.5[3.4]
	5	36.8[3.4]	34.6[3.7]	36.5[3.7]	38.5[3.4]
	F	42.1[4.2]	41.1[4.7]	39.1[4.7]	42.5[4.1]
RIA (%)	3	0.84[0.52]^{a}	0.49[0.51]^{a}	2.83[0.73]^{b}	4.24[0.60]^{b}
	4	1.66[0.50]^{a}	1.31[0.49]^{a}	3.65[0.72]^{b}	5.06[0.58]^{b}
	5	1.88[0.46]^{a}	1.52[0.47]^{a}	3.86[0.70]^{b}	5.27[0.57]^{b}
	F	3.52[0.65]^{a}	3.92[0.87]^{ab}	4.79[0.87]^{ab}	5.98[0.63]^{b}
EMG Onset-Curvature Phase Lag (%)	3	1.60[2.49]^{a}	0.61[2.88]^{a}	-0.47[2.20]^{a}	-2.80[2.02]^{a}
	4	-6.72[2.45]^{a}	-7.71[2.84]^{a}	-8.79[2.13]^{a}	-11.12[1.96]^{a}
	5	-4.70[2.40]^{a}	-5.69[2.84]^{a}	-6.77[2.12]^{a}	-9.10[1.95]^{a}

Report is given only for the left-side electrode positions; values for right-side electrodes at positions 2 and 4 are comparable to left-side electrodes 2 and 4. H, head; T, tail; F, right pectoral fin. Estimated marginal means are presented with their standard error (calculated based on the number of trials in each viscosity) in square brackets. Connecting letters are in superscript following the standard error. Position is the position on the body of the fish and was included in models only for those variables where data were quantified at multiple positions.

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2.9 Supplementary Material

Supplementary material (R code for statistical analysis, dataset, Movie 2.1, and Movie 2.2) is available here.

Movie 2.1. *Polypterus senegalus* swimming in normal water. Video was filmed at 250 fps. Play back is at 60 fps.

Movie 2.2. *Polypterus senegalus* swimming in 40 cP water. Video was filmed at 250 fps. Play back is at 60 fps.

CHAPTER 3: BEHAVIOUR AND MUSCLE ACTIVITY ACROSS THE AQUATIC-TERRESTRIAL TRANSITION IN *POLYPTERUS SENEGALUS*

Chapter Notes

This chapter is currently under review:

Lutek, K., Foster, K.L. and Standen, E.M. Behaviour and muscle activity across the aquatic-terrestrial transition in *Polypterus senegalus*. *J. Exp. Biol.*

Data for the 'kinematics set' was collected and analyzed by K.L. Data for the 'EMG set' was collected by K.L.F. and analyzed by K.L.

Author contributions

Conceptualization: K.L., K.L.F., E.M.S.; Methodology: K.L., K.L.F., E.M.S.; Software: K.L., E.M.S.; Validation: K.L., K.L.F., E.M.S.; Formal Analysis: K.L.; Investigation: K.L., K.L.F.; Resources: E.M.S.; Data Curation: K.L., E.M.S.; Writing – original draft: K.L.; Writing – review editing: K.L., K.L.F., E.M.S.; Visualization: K.L.; Supervision: E.M.S.; Project Administration: E.M.S.; Funding Acquisition: E.M.S.

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3.1 Abstract

Amphibious fishes moving from water to land experience continuous changes in environmental forces. How these subtle changes impact behavioural transitions cannot be resolved by comparisons of aquatic and terrestrial locomotion. For example, aquatic and terrestrial locomotion appear distinct in the actinopterygian fish *Polypterus senegalus*; however, it is unclear how gradual water level changes influence this locomotor transition. We test the hypothesis in *P. senegalus* that swimming and walking are part of an incremental behavioural and muscle activity continuum across the environmental transition from water to land, rather than two discrete behaviours as proposed by previous literature. We exposed *P. senegalus* to discrete environments from fully aquatic to fully terrestrial while recording body and pectoral fin kinematics and muscle activity. Anterior axial red muscle effort increases as water depth decreases, however, an anterior-to-posterior wave of axial red muscle activity is always present, indicating gradual motor control changes. Thus, walking appears to be based on swimming-like axial muscle activity while kinematic differences between swimming and walking follow environmental mechanical constraints. A discrete change in pectoral fin coordination from in-phase to out-of-phase at 0.7 body depths relies on adductor muscle activity with a similar duty factor and adductor muscle effort that increases gradually as water depth decreases. Thus, despite altered coordination timing, neuromuscular patterning is similar. Since the observed gradual axial muscle effort increases reflect muscle activity changes between aquatic and terrestrial environments for other elongate fishes, a modified, swimming-like axial muscle activity pattern for terrestrial locomotion may be common among elongate amphibious fishes.

3.2 Introduction

Amphibious fishes move through disparate physical environments using a single locomotor control system (central pattern generators, musculoskeletal elements, and sensory systems). The locomotor control system in fishes is adapted for an aquatic environment. To facilitate terrestrial movement, some species have evolved specialized structures (e.g., pectoral fins in mudskippers; Harris, 1960; Murdy, 1989) while others rely only on the locomotor flexibility of unspecialized morphology (e.g., *Polypterus senegalus* and *Kryptolebias marmoratus*; Foster et al., 2018; Perlman and Ashley-Ross, 2016; Pronko et al., 2013; Standen et al., 2014; Standen et al., 2016).

While many papers investigate how fishes move in a terrestrial environment (reviewed in Pace and Gibb, 2014 and Lutek et al., 2022), less is known about how motor control and sensory information elicit terrestrial behaviour in amphibious animals (Cabelguen et al., 2010; Foster et al., 2018; Gillis, 2000; Horner and Jayne, 2014; Perlman and Ashley-Ross, 2016). Studies that investigate fish muscle activation in fully aquatic or terrestrial environments often report statistically significant differences in muscle activity parameters between the two environments (Foster et al., 2018; Gillis, 2000; Horner and Jayne, 2014; Perlman and Ashley-Ross, 2016). In general, terrestrial axial muscle activity has a higher intensity and longer duration and the adductor of the pectoral fin may change from a single burst pattern during swimming to a double burst pattern during walking (Foster et al., 2018; Gillis, 2000; Horner and Jayne, 2014; Perlman and Ashley-Ross, 2016). Thus, terrestrial locomotion in amphibious fishes appears to require more muscle effort, likely to overcome the increase in friction between the body and substrate and decrease in buoyancy from the water that accompanies the transition to land (Fig. 3.1A,C). While these studies speak to the discrete difference between fully aquatic and fully terrestrial biomechanics, they do not address the possibility of a behavioural continuum across the ecologically relevant transition between water and land. Subtle

changes in water level will alter environmental physical forces and resultant sensory feedback, which help shape motor control and resultant behaviour (Fig 3.1A,B,C).

Few studies have investigated the kinematics of the aquatic-terrestrial transition in amphibious animals. Salamanders moving up or down a slope switch between walking and swimming while partially submerged, but precisely when this transition occurs varies between trials (Ashley-Ross and Bechtel, 2004). Elongate *Erpetoichthys calabaricus* (ropefish), when in intermediate water depths, move using behaviour that combines the characteristics of aquatic and terrestrial locomotion (Pace and Gibb, 2011). The more classically 'fusiform' *K. marmoratus* (mangrove rivulus) use three separate behaviours (launches, squiggles, and pounces) to move between aquatic and terrestrial environments, depending on the physical environment and desired outcome (Pronko et al., 2013). Each species navigates the aquatic-terrestrial transition in a unique way, possibly due to differences in which propulsive elements are used for locomotion. For example, salamanders swim using axial undulations and walk using their legs (Cabelguen et al., 2003) while ropefish use primarily axial undulations to move in both aquatic and terrestrial environments (Pace and Gibb, 2011). Determining how, and in which environments, body and fin motion and muscle activity change sheds light on the complex interactions of axial/appendicular motor control and the physical environment in amphibious animals.

Polypterus senegalus is the extant species closest to the common ancestor of actinopterygians and sarcopterygians and is capable of terrestrial locomotion powered by coordinated pectoral fin and body movements. Previous work demonstrates marked kinematic and muscle activity differences between swimming and walking in this species (Foster et al., 2018; Standen et al., 2014; Standen et al., 2016). During swimming, pectoral fins are moved in-phase with little evidence of adductor/abductor co-contraction (Foster et al., 2018; Standen et al., 2014; Standen et al., 2016); body undulation is minimal with little or no red muscle activity in the anterior half of the body.

When moving over land (i.e. walking), pectoral fins move out-of-phase with a large range of motion (RoM) and the body exhibits large lateral undulations (Standen et al., 2014; Standen et al., 2016). These movements are powered by activation of the adductor muscle in the pectoral fin that suggests co-contraction with the abductor, likely leading to fin stiffening, and increased red muscle recruitment at the middle of the body (Foster et al., 2018). However, these investigations looked at behaviour only at the extremes of the aquatic-terrestrial environmental continuum. Thus, they also do not address the possibility of a gradual behavioural or control transition between swimming and walking that varies with an environmental gradient.

To further investigate the swimming to walking transition, we exposed *P. senegalus* to stepwise water depths that span the aquatic-terrestrial transition. To capture a finer resolution across those environments where changes in physical forces within the environment are largest, we pay particular attention to the depths at which buoyancy likely gives way to gravity as the dominant force (Fig 3.1B). In this way we test the hypothesis that the transition from swimming to walking in *P. senegalus* is actually a continuum (the 'continuum hypothesis') and predict that as water depth decreases there will be a gradual shift in kinematics and motor control patterns. The continuum hypothesis exists in contrast to the alternative hypothesis (the 'discrete hypothesis'), which, based on the existing dichotomous literature, posits that there is a discontinuity between walking and swimming and predicts that a discreet switch exists in both kinematics and muscle activation pattern at a critical water depth.

3.3 Materials and Methods

3.3.1 Animals

The results presented here are from two experiments on two different groups of fishes. In one group of fishes (kinematics set; n=5) we recorded kinematics across a gradient of eight water depth treatments, spanning fully aquatic to fully terrestrial, measured as a proportion of body depth

(BD): 3.0 BD, 2.0 BD, 1.1 BD, 1.0 BD, 0.9 BD, 0.7 BD (height of the top of the eyes), 0.1 BD (height of the mouth) and 0.0 BD (no water above the wetted substrate) over the course of several days (Movies 3.1, 3.2, 3.3). Body depth was standardized as the distance from the ground to the tallest part of the dorsal surface (not including the dorsal fin) of each fish. The kinematics set had an average total body length (BL) of 95.5 ± 3.20 mm and an average mass of 5.01 ± 0.54 g. Each fish in the kinematics set was filmed in no more than three conditions each day and no more than three days each week to avoid fatigue effects. In a second group of fishes (electromyography [EMG] set; n=4; BL: 123.5 ± 3.66 mm; mass: 9.29 ± 0.89 g), we recorded kinematics and muscle activity in depths of 3.0 BD, 1.1 BD, 1.0 BD, 0.9 BD, and 0.7 BD (Fig. 3.1). We were limited to these depths since EMG experiments in *P. senegalus* must be completed in a single day. Each fish in the EMG set was recorded in order from greatest to least BD and permitted time to rest between conditions and bouts of filming. Unless otherwise stated, methods are applicable for both sets of animals and all values are reported as a mean ± s.e.m.

Polypterus senegalus were acquired from the pet trade (AQUAlity Tropical Fish Wholesale Inc., Mississauga, ON, Canada) and housed in individual recirculating (10% water change each week) tanks on a 12/12 light cycle at 25-26 °C. Except for one fish from the kinematics set in 0.1 BD (which completed three cycles), all fishes completed at least five tailbeat cycles at each water depth. Experiments were performed according to the University of Ottawa Animal Care Protocol BL-1926.

3.3.2 High-Speed Videography

For the kinematics set, trials were filmed on a 21 x 10 cm plexiglass board covered tightly in cheesecloth to increase traction and placed in a 38 L tank. High speed video (500 fps) was captured from a single side view (Fastec IL5, Fastec Imaging San Diego CA, USA) and two top views, using two Photron Fastcam Mini UX (Photron USA Inc., San Diego, CA, USA) suspended above the platform and angled approximately 45° from each other. These three camera views were calibrated

for three-dimensional (3D) analysis using a 42-point calibration object in DLTcal5 (Hedrick, 2008), a custom Matlab (Version R2018/2019/2020, The Mathworks Inc., Nantick, MA, USA) digitization program.

For the EMG set, trials were filmed on a 35 x 35 cm plexiglass board covered tightly in cheesecloth and placed in a 34 L tank. High speed video (500 fps) was captured from a top view (Photron Fastcam) and three side views (one Photron Fastcam and two Fastec IL5). These four camera views were calibrated for 3D analysis using a 44-point calibration object in DLTcal5 (Hendrick, 2008).

In all videos, the nose, tail tip, and middle of the distal edge of the pectoral fin lobe for the right fin were digitized manually in DLTdv6 (Hedrick, 2008) to get 3D coordinates. For the kinematics set, the left fin lobe tip was also digitized as above to calculate fin RoM. For both datasets, top views were binarized and fish midlines were tracked automatically using custom Matlab code. One hundred evenly spaced points along the fish's body were selected from these midlines and used to reconstruct the fish midline coordinates in the x-y plane. Timing of the beginning of fin adduction and abduction was identified manually in the open-source image analysis platform, FIJI (Schindelin et al., 2012).

3.3.3 Kinematics Analysis

To facilitate comparisons across water depths, stroke timing was calculated two ways: 1) as pectoral fin stroke, defined by right pectoral fin motion (start of adduction = stroke start, start of abduction = mid-stroke) and 2) tail stroke, defined by tail motion (start of tail swing to the right = stroke start, start of tail swing to the left = mid-stroke). Since swimming *P. senegalus* show no coordination between body and pectoral fins both in previous and present datasets (Foster et al., 2018; Standen et al., 2014), left fin timing was defined relative to the pectoral fin stroke and timing of maximum left and right amplitude for the body was defined relative to the tail stroke.

For each trial, we calculated the following variables: locomotion speed (BL s-1), curvature coefficient (a measure of overall fish curvature; BL), swing distance (total distance a body point travels in the x-y plane from maximum left amplitude to maximum right amplitude; BL), wave frequency (cycles s-1), pectoral fin frequency (cycles s-1), pectoral fin RoM (maximum fin angle – minimum fin angle; degrees) and nose elevation (BL). Magnitude variables were standardized to BL to permit comparisons between individuals. Curvature coefficient (modified from Brainerd and Patek, 1998) was calculated as follows:

$$1 - d(n,t) \tag{3.1}$$

In Eqn 3.1, $d(n,t)$ is the minimum linear distance between nose and tail during a locomotor cycle standardized to BL. Thus, a value of 1 represents the nose and tail touching during locomotion and a value of 0 represents a perfectly straight fish. Pectoral fin frequency was calculated based on the pectoral fin stroke as defined above. Pectoral fin angle was defined as the angle between the nose, back of skull and tip of fin lobe (as in Foster et al., 2018 and Lutek and Standen, 2021). Both fin RoM and nose elevation showed no differences in magnitude between left and right side, thus a mean value for each trial calculated from both left and right side data was used in the final analysis. All linear variables use trial averages as individual observations. We report the start-time of left fin adduction, and the timing of maximum amplitude of the body to the left and to the right in polar coordinates. Maximum body amplitude timing is assessed only at 40%, 60% and 80% BL since body amplitude is minimal at more anterior positions in higher water depths. Since polar coordinates are relative and circular, they facilitate focus on the phase differences between strokes. All variables were calculated using custom code in Matlab. Since the kinematics of both experimental sets of fishes were similar, we report only the kinematics set for these measures, as this set covers the largest number of water depths.

3.3.4 Electromyography

For the EMG set, each fish was anaesthetized prior to surgery using 200 mg L^{-1} buffered tricane methanesulfonate (MS222, Syndel Laboratories Ltd. BC, Canada). Throughout surgery fishes were kept moist and sedated using fish housing water or anaesthetic as required. Two-pronged fine wire hook electrodes were fashioned from 0.051 mm bi-filament wire (California Fine Wire Company, CA, USA) and implanted percutaneously using 27-gauge needles (Sigma-Aldrich, MO, USA). Electrodes were implanted in the middle of the pectoral fin adductor, in five locations spaced evenly along the left side red body muscle between the pectoral fin lobe and anal fin (19.8 ± 0.73% BL, 33.1 ± 0.51% BL, 42.8 ± 0.69% BL, 57.4 ± 0.39% BL and 67.4 ± 0.74% BL), and opposite positions 2 and 4 on the right side of the body (as in Lutek and Standen, 2021). Following the placement of all electrodes, the wires were sutured to the dorsal spines to reduce strain on the electrodes. Fish recovered in fish housing water prior to performing locomotor trials. We recorded EMG signals using a Grass P511 AC Amplifier (Natus Neurology, CA, USA) passed through a Powerlab 16/35 digital-to-analog converter (AD Instruments, CO, USA). Signals were recorded at 10 kHz with a 60 Hz notch filter (to eliminate ambient electrical noise) in Lab Chart 8 (AD Instruments, CO, USA). Signals were then filtered with a 40-4000 Hz bandpass filter and analyzed using custom Matlab code. Video and EMG recordings were synchronized using an external trigger. Following recordings in all depths, each fish was euthanized with an overdose of buffered MS222 (417 mg L^{-1} in fish housing water). Then mass and BL were recorded and electrode position was confirmed by dissection.

Electromyography Analysis

Muscle burst start and stop times were identified from EMG traces manually. Note that two fishes in 0.7 BD did not have consistent bursts of muscle activity and so onsets and offsets were not identified for these individuals (Fig. S3.1). Using the onset and offset timings, we calculated burst

duration as a percentage of the tailbeat cycle (EMG duty factor) and rectified integrated area (EMG RIA; the sum of rectified muscle signals within a burst of activity reported as a percentage of the theoretical maximum RIA to permit comparison between individuals). This theoretical maximum was calculated as the mean of the top 5% of muscle signals for a given electrode within each fish multiplied by the duration of an individual burst of muscle activity. EMG duty factor and EMG RIA showed no difference between left-side and right-side electrode positions and so are reported only for the left-side electrodes. Timing of body EMG onsets and offsets for electrodes on the left side of the body at positions 3, 4, and 5 are reported relative to the tailbeat cycle in polar coordinates. We looked only at these sites to match the body sites assessed for consistent maximum kinematic amplitude timing. Contralateral co-activation of left and right side muscles was quantified by comparing timing of muscle bursts at the same position on opposite sides of the body (electrode sites 2 and 4).

3.3.5 Statistical Analysis

All linear variables were analyzed using linear mixed effects models created in R using the nlme package (Pinheiro et al., 2018; R Team Core, 2018). Models for each linear variable included fixed effects for swim speed, water depth, and site (as dictated by model comparisons) and fish identity as a random effect (full model fixed effects: dependant variable ~ speed * depth * site, random effects: ~1 | fish) (see Table S3.1 for specific models for each dependant variable). Residuals were checked for normality (visual inspection of a histogram) and equal variance (Levene's Test) across water depths. When necessary, models were adjusted to accommodate unequal variance across water depth or site using the varIdent function (Pinheiro et al., 2018; Table S1). ANOVA-type output tables (Type III Sums of Squares) were generated using the anova.lme function (Pinheiro et al., 2018). R-squared values were estimated using the MuMIn package (Barton, 2018). Pairwise multiple comparisons were performed as appropriate based on the estimated marginal

means (and associated s.e.m.) from each model. These pairwise comparisons were Bonferroni-corrected based on the number of comparisons performed within each kinematic variable to account for type I error.

Cycle timing (polar coordinates) was analyzed using standard polar statistics (similar to Standen, 2008; Standen et al., 2014) using custom Matlab code and the Circular Statistics Toolbox (Berens, 2009) based on Zar (1999) and Batschelet (1981). First, data were tested for a non-uniform distribution using the Hermans-Rasson (HR) test (Landler et al., 2019). This test is sensitive to any deviation from a uniform distribution, including non-axial, bimodal distributions (as observed in 0.7 and 0.1 BD for left pectoral fin adduction timing in our dataset). If a variable was non-uniform (HR p-value < 0.05), it was then tested for a von Mises distribution. The von Mises distribution is the circular distribution analog of the linear normal distribution and is a requirement for running Rayleigh's test for a unimodal distribution. Thus, if the data had a von Mises distribution, data were tested for a unimodal distribution using Rayleigh's test. The combination of the HR and Rayleigh's test in this order distinguishes between non-uniform, multi modal distributions (HR p-value < 0.05, Rayleigh's test p-value > 0.05) and non-uniform, unimodal distributions (HR p-value < 0.05, Rayleigh's test p-value < 0.05). A non-uniform, multi modal distribution suggests that data timing occurs consistently at multiple time points in a kinematic cycle (e.g. an event that occurs twice with regular timing during a kinematic cycle). A non-uniform, unimodal distribution suggests that data timing occurs consistently at a single time point in the kinematic cycle. If the data had a non-uniform, unimodal distribution, an angular mean and angular variance were calculated. No angular mean or angular variance was calculated for multi-modal distributions.

We additionally investigated whether left pectoral fin adduction timing was consistent within each trial by testing for a von Mises distribution, a non-uniform unimodal distribution (Rayleigh's test) and calculating a mean and angular variance when the Rayleigh's test p-value was < 0.05. For

nose elevation, we tested for differences in angular dispersion (the absolute difference between each observation and the circular mean in each depth) between water depths using a Kruskal-Wallis test to determine whether kinematic timing became more or less variable across water depths (Zar, 1999). For the timing of maximum body amplitude and the timing of body muscle onset and offset, we tested for differences in angular mean between water depths using Watson-Williams tests (Zar, 1999). Both Kruskal-Wallis and Watson-Williams tests were followed by pairwise comparisons as needed. These pairwise comparisons were Bonferroni-corrected (as for linear statistics above) to account for type I error.

3.4 Results

3.4.1 Kinematic changes across water depths

Mean locomotion speed was consistent across water depths with a slight increase at 0.9 BD and a slight decrease at 0.1 BD and 0.0 BD relative to other depths (Tables 3.1, 3.2). Curvature coefficient increased as water depth decreased below 1.0 BD (Tables 3.1, 3.2, Fig. 3.2A). Swing distance increased significantly with speed and towards the posterior of the fish (Tables 3.1, 3.3). Swing distance tended to increase as water depth decreased (Tables 3.1, 3.2). Wave frequency increased significantly as fish moved faster (Table 3.1). There was no difference in wave frequency across sites (Tables 3.1, 3.3). Across water depths, wave frequency was significantly higher at 1.1 BD, 0.7 BD and 0.1 BD than at 3.0 BD (Tables 3.1, 3.2). The effect of locomotion speed on pectoral fin frequency depended on depth (Table 3.1). Pectoral fin frequency increased slightly between 3.0 and 0.9 BD, then decreased as water depth dropped to 0.0 BD (Tables 3.1, 3.2). Pectoral fin range of motion increased significantly as water depth decreased below 1.0 BD and increased significantly again as water depth decreased below 0.1 BD (Tables 3.1, 3.2, Fig. 3.2B). Nose elevation became significantly larger in 0.1 BD and 0.0 BD (Tables 3.1, 3.2, Fig. 3.3A).

In general, the timing of the left pectoral fin relative to the right pectoral fin was either in- or out-of-phase, with few strokes having an intermediate timing (Fig. 3.4A). Pectoral fin timing was non-uniform (HRp < 0.0001) and unimodal (Rp <0.05) in all conditions except 0.7 BD and 0.1 BD (Rp > 0.05; Fig. 3.4A) where trials had consistent timing (Fig. S3.2) but were both in- and out-of-phase in these conditions, sometimes within an individual fish. For trials that were too short or had variable timing, and thus had a p-value from Rayleigh's test larger than 0.05, individual data are still shown, but no mean or confidence interval could be calculated for these data (Fig. S3.2). In 3.0 and 2.0 BD, the timing of leftward maximum body amplitude was consistent, coincident with maximum leftward tail amplitude for 40% BL, just before maximum rightward tail amplitude for 60% BL and just after maximum rightward tail amplitude for 80% BL (Table 3.4, Fig. 3.4B). Likewise for the timing of rightward maximum body amplitude relative to maximum leftward tail amplitude. The timing of maximum leftward and rightward amplitude at all positions shifts significantly later in the cycle at 1.1 BD, compared to 3.0 and 2.0 BD, and maintains this timing in 1.0 BD (Table 3.4, Fig. 3.4B). Maximum leftward and rightward amplitude at all positions shift later again in 0.9 BD, relative to 1.1 and 1.0 BD, and remain consistent through 0.1 BD (Table 3.4, Fig. 3.4B). In 0.0 BD, the timing of maximum leftward and rightward amplitude at 40% and 60% BL are consistent with timing in 0.9 through 0.1 BD, but the timing of 80% BL shifts later in the cycle again, relative to 0.9 through 0.1 BD. The timing of maximum nose elevation, relative to the pectoral fin stroke cycle, was directional (just after the beginning of the stroke) in 0.9 BD (nose elevation to the right only), 0.7 BD, 0.1 BD and 0.0 BD (Fig. 3.3B). The angular dispersion of nose elevation decreased as water depth decreased for nose elevation to the right (X^2= 41.45; df=5; p<0.0001) and to the left (X^2= 50.42; df = 5; p<0.0001) (Fig. 3.3B). Dispersion was significantly lower in 0.1 BD (nose elevation to the left) and 0.0 BD (nose elevation to the right and left) than at 0.9 BD and above (Fig. 3.3B).

3.4.2 Muscle activity changes across water depths

EMG RIA at body positions 1 and 2 and in the adductor muscle of the pectoral fin increased significantly as water depth decreased but showed no significant changes at body positions 3-5 (Tables 3.1, 3.2, 3.3, Fig. 3.5A). EMG duty factor remained constant across all fin and body electrodes and in all treatments (Tables 3.1, 3.2, 3.3, Fig. 3.5B). Note, however, that two individuals had muscle activity in the adductor of the pectoral fin that was distinctly different (reminiscent of walking traces reported previously; Foster et al., 2018) and showed inconsistent and indistinct muscle activity in 0.7 BD (Fig. S3.1). The timing of red axial body muscle onset along the left side of the body at sites 3, 4 and 5 generally shifted later in the tailbeat cycle as depth decreased (Table 3.4, Fig. 3.5C). Right and left side body muscle was rarely active at the same time. At position 2, right and left sides were active simultaneously for the following number of bursts in each condition, 3.0 BD – 0/35, 1.1 BD – 2/49, 1.0 BD – 4/38, 0.9 BD – 5/37, 0.7 BD – 2/41, and for position 4, 3.0 BD – 0/45, 1.1 BD – 1/52, 1.0 BD – 0/44, 0.9 BD – 2/37, 0.7 BD – 2/42.

3.5 Discussion

3.5.1 Aquatic and terrestrial locomotion in elongate fishes

Polypterus senegalus exhibited discrete kinematic changes at water depths where the body and pectoral fin were first obligated to interact with the substrate. Despite this, axial red muscle always carried a travelling wave of muscle activation from anterior to posterior with little contralateral muscle activity overlap. Additionally, muscle effort gradually increased as water depth decreased. These results are not entirely in support of either the 'discrete' or the 'continuum hypothesis'. Rather, it appears that the body and pectoral fins of *P. senegalus* change across the aquatic-terrestrial transition in different ways. Since red axial muscle always carried an anterior-to-posterior wave of muscle activity and increases in muscle effort were continuous, we suggest that walking in *P. senegalus* is accomplished by swimming-like body muscle activity. The different kinematic output between

swimming and walking can be explained because, although the muscle activity pattern is similar, interactions between the fish and the external environment constrain axial red muscle contractions, resulting in modified kinematic performance. The increased friction and lack of buoyancy in transitional environments may change the range of muscle lengths over which axial muscles are active and/or alter the terrestrial waveform by physically hindering kinematic movement. Thus, changes in axial muscle activity across the aquatic-terrestrial transition support the 'continuum hypothesis'.

Between swimming and walking modes the pectoral fins show a starker contrast in behaviour. As fish move into shallow water (0.7 and 0.1 BD) their left and right pectoral fins switch discretely from in-phase to out-of-phase movements and the adductor muscle of the pectoral fin undergoes a much longer activation duration in seconds because pectoral fin beat frequency is lower during walking than swimming (Tables 3.1, 3.2). This does not necessarily suggest that a different neural circuit is used for fin motion during walking. Here, increases in pectoral fin muscle activation duration are not accompanied by a change in EMG duty factor, meaning the timing of individual pectoral fin patterns within the step cycle is similar between most walking steps (see 'Mechanisms of muscle modulation' below for details) and swimming strokes. In addition, asymmetric pectoral fin coordination is common during unsteady (e.g., turning, acceleration, backwards swimming) aquatic behaviours both in *P. senegalus* (K. Lutek and E.M. Standen unpublished observations) and other fish species (e.g. Drucker and Lauder, 2002; Drucker and Lauder, 2003; Gerstner, 1999), suggesting this is not a novel terrestrial control pattern. Additionally, a discrete switch between limb use and limb adduction against the body is seen in both live and modeled salamander locomotion and may be due to a single control pattern (Cabelguen et al., 2003; Ijspeert et al., 2007). A single neural circuit may elicit both walking and swimming in salamanders if limbs are tonically adducted above a threshold descending neural drive (Ijspeert et al., 2007). Thus, although there is a large and apparently discreet

change in the left-right coordination of pectoral fin kinematics at particular water depths, this may be driven by a gradual increase in descending drive, and a particular neural threshold that switches pectoral fin movements from in-phase to out-of-phase. The gradual increase in drive could, for example, be driven by sensory feedback based on changes in the relative magnitudes of gravitational and buoyant forces across the environmental continuum. If an interneuron changes its firing pattern (or starts firing) above a given feedback threshold, this could elicit a change in coordination of the pectoral fins. If pectoral fin kinematic changes are driven by a single neural circuit with appropriate neural thresholds, as we suggest and as observed in salamanders, then despite a discrete change in outward kinematics, these results would be consistent with the 'continuum hypothesis'.

How does this view of walking compare to terrestrial muscle activity for other elongate fishes? Comparisons of muscle activity between aquatic and terrestrial locomotion in *P. senegalus*, in eels, and in lungfish show similar changes in muscle activity (Foster et al., 2018; Gillis, 2000; Horner and Jayne, 2014). All three species tend to have higher anterior body RIA, longer body EMG duration, and body EMG onset later in the kinematic cycle during terrestrial locomotion. Our study shows similar muscle activity changes, but in a gradual manner as water depth decreases. This raises the possibility that these three species accomplish terrestrial locomotion using a swimming-like axial muscle activity pattern that is highly influenced by interaction with the substrate.

While this is the first report of fish muscle activity in environments that span the water-land interface, previous kinematic work in the elongate ropefish provides an interesting point of comparison for our results. Both aquatic and terrestrial locomotion in ropefish is primarily powered by body movements. In contrast, *P. senegalus* power swimming primarily with pectoral fin movements (and gradually recruit body undulations as swimming speed increases) and both pectoral fins and body power terrestrial locomotion. Despite this difference, both species appear to show an increase in body curvature with decreasing water depth (Pace and Gibb, 2011). Although EMG is

not recorded for ropefish transitioning through different water depths, the ropefish kinematic response is like *P. senegalus* and suggests that ropefish may also use a swimming-like axial muscle activity pattern during terrestrial locomotion. Ultimately, investigating muscle activity across the aquatic-terrestrial transition in multiple species, including water depths that bridge the gap between 0.7 BD and 0.0 BD in *P. senegalus*, is essential to test the 'continuum hypothesis' across these amphibious fishes.

3.5.2 Mechanisms of muscle activity modulation

That a fish can have a discreet change in behaviour with a continuous change in muscle activation emphasizes the role of environmental constraint on the resulting kinematics. Compared to swimming, body bending and fin range of motion increased significantly and peak body bending occurred later in the tailbeat cycle when water dropped to 0.9 BD. This coincides with the environment where the weight of the fish is no longer fully supported by buoyant forces (Fig. 3.1B). This increase in body and fin motion is likely required to increase locomotor force and overcome higher resistance resulting from the slow increase in frictional loading of the belly on the ground. As water depth decreased further, to a point where the pectoral fin must interact with the substrate (0.7 BD), fin kinematic timing changed from in-phase to out-of-phase oscillations. The switch to out-of-phase pectoral fin movements allowed fins to coordinate with the increase in body curvature helping to lift the anterior body off the ground (Figs 3.2, 3.3). This likely created higher ground reaction forces to facilitate lift and forward movement. Presumably, there is a threshold water depth below which thrust production using in-phase fin movements can no longer overcome the increased ground friction. If *P. senegalus* provide propulsion for steady locomotion using either in- or out-of-phase fin movements, the discrete use of these behaviours in different environments could facilitate the most effective locomotion based on the physical limitations of each environment (Fig. 3.6A,B). The torque and thrust generated by in-phase pectoral fin movements in an aquatic environment

would be balanced, while that generated by out-of-phase movements would generate roll and lateral movement of the nose (Fig. 3.6A). These unbalanced forces would likely decrease swimming efficiency. In contrast, in-phase pectoral fin movements in water depths <0.7 BD cannot lift the body of *P. senegalus* off the ground, thus rendering them ineffective for terrestrial locomotion in this species (Fig. 3.6B). Out-of-phase fin movements in a terrestrial environment allow the body of the fish to roll and thus the head to lift off the ground, reducing friction between the body and substrate (Fig. 3.6B). Partitioning locomotor modes based on the ability to produce forward movement through a particular environment may be a common characteristic of transitional movements in amphibious fishes. *K. marmoratus* also appear to select particular transitional behaviours based on the environment they are in (Pronko et al., 2013) and ropefish have individual trials that are more swimming- or walking-like when 75% emersed, potentially due to microfluctuations in the testing environment (Pace and Gibb, 2011). A discrete separation of locomotor strategies in these transitional environments is likely the result of a set of complex (and possibly conflicting) physical constraints and sensory cues and such behavioural switches may be easily accomplished using neural thresholds (in line with the 'continuum hypothesis') as described above.

Environmental constraint combined with subtle antagonistic muscle activation may also explain how two different muscle activity patterns in the adductor of the pectoral fin at 0.7 BD (Fig. S3.1) result in a single kinematic function. One pattern exhibits a more continuous activation of the adductor muscle while the other shows clear independent bursts, but both patterns are associated with a clear step cycle for the fin. For a continuous muscle contraction to be active during both adduction and abduction in a fin cycle, the muscle must operate across both lengthening and shortening regimes. This means that the muscle is working against the constraint of the environment, lengthening under the weight of the body against the ground, or working against the contraction of antagonistic muscles of the fin. The occurrence of two muscle activation patterns that

result in the same kinematic output suggests that fish can employ multiple 'strategies' to achieve the same kinematic function. Thus, effective locomotion here is the result of an interaction between the existing musculoskeletal anatomy of the animal and the physical constraints of the environment itself, which confines outward behaviour to a single successful kinematic function. It is interesting to speculate that because the extended muscle activity duration in the adductor of the pectoral fin that we observed occasionally in 0.7 BD is reminiscent of 'double' bursts reported previously during walking (Foster et al., 2018), at water depths below 0.7 BD the adductor muscle of the pectoral fin may always behave in this extended duration capacity.

3.5.3 Neuromuscular control in novel environments

Since a swimming-like axial motor pattern appears to be sufficient to produce walking in *P. senegalus*, this suggests the utility (and possible evolutionary advantage) of a single pattern that can be used in several environments. *Polypterus senegalus* also use modified swimming axial muscle activity in high viscosity (Lutek and Standen, 2021). However, in such high resistance aquatic environments, they increase posterior body muscle effort (rather than anterior body muscle effort as water depth decreases), suggesting different modifications of the base swimming pattern in response to novel aquatic and terrestrial environments. If the same motor control circuit that coordinates swimming can be used in more resistive environments, regardless of medium, then exploring novel environments becomes easier, even when there is no prior exposure to that environment (as seems likely the case for land in *P. senegalus*; Du et al., 2016). That the body and pectoral fins change kinematic timing in different depths (at 0.9 BD and 0.7 BD, respectively) may also suggest a weak neural coupling between the two. This may afford these fish additional flexibility in their behavioural repertoire by allowing independent modulation of the body and pectoral fins. Overall, our results suggest that in *P. senegalus* axial and pectoral fin muscle activity across the aquatic-terrestrial transition is consistent with the 'continuum hypothesis' and that discrete changes in outward

kinematics are the result of physical environmental constraint acting on propulsive structures. Further investigations of amphibious fishes in environmental gradients, such as we have done here, will help determine whether this principle holds across a variety of fishes, or if this control strategy is unique to *P. senegalus*.

3.6 Figures & Tables

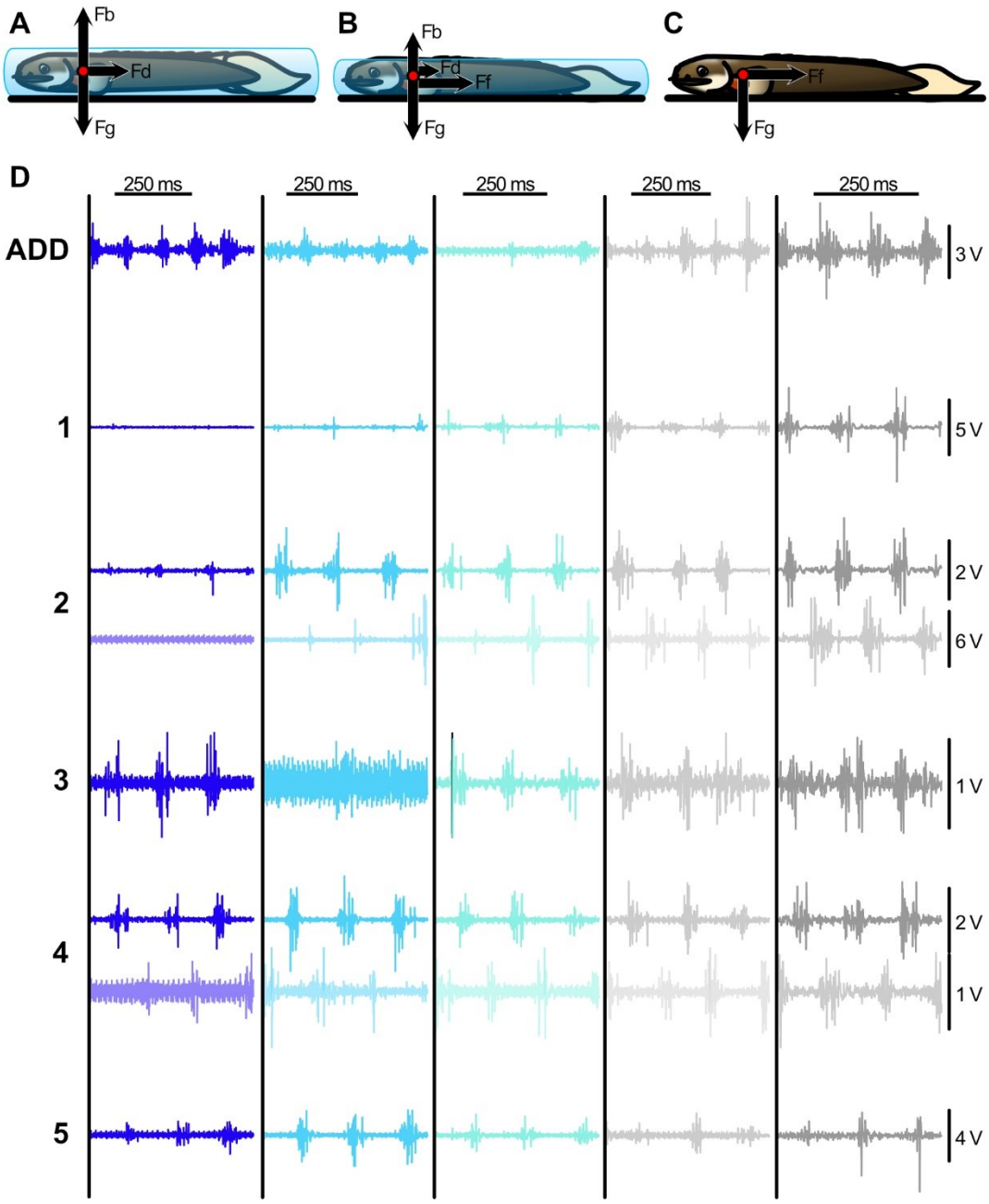

Figure 3.1. Physical forces and example EMG traces for *P. senegalus* across the aquatic-terrestrial transition. Free body diagram for *P. senegalus* in (A) 1.1 BD, (B) in 0.9 BD, and (C) 0.0 BD. For simplicity, we assume the center of buoyancy and center of gravity are in the same position on the fish and all force vectors are drawn as acting at this point. Arrow size represents force magnitude, but these magnitudes are not scaled. Thus, differences in arrow size are qualitative not quantitative. (D) Example EMG traces for a *P. senegalus* moving in water depths of 3.0 BD, 1.1 BD, 1.0 BD, 0.9 BD and 0.7 BD (from left to right). Electrodes in the adductor muscle of the pectoral fin (ADD) and along the left side of the body are shown in full colour. Electrodes along the right

side of the body are shown directly below in lighter shades. 1 through 5 are positions spaced equidistantly in red axial muscle from the posterior edge of the pectoral fin lobe to the anterior edge of the anal fin. See text for precise location. All traces are from a single individual.

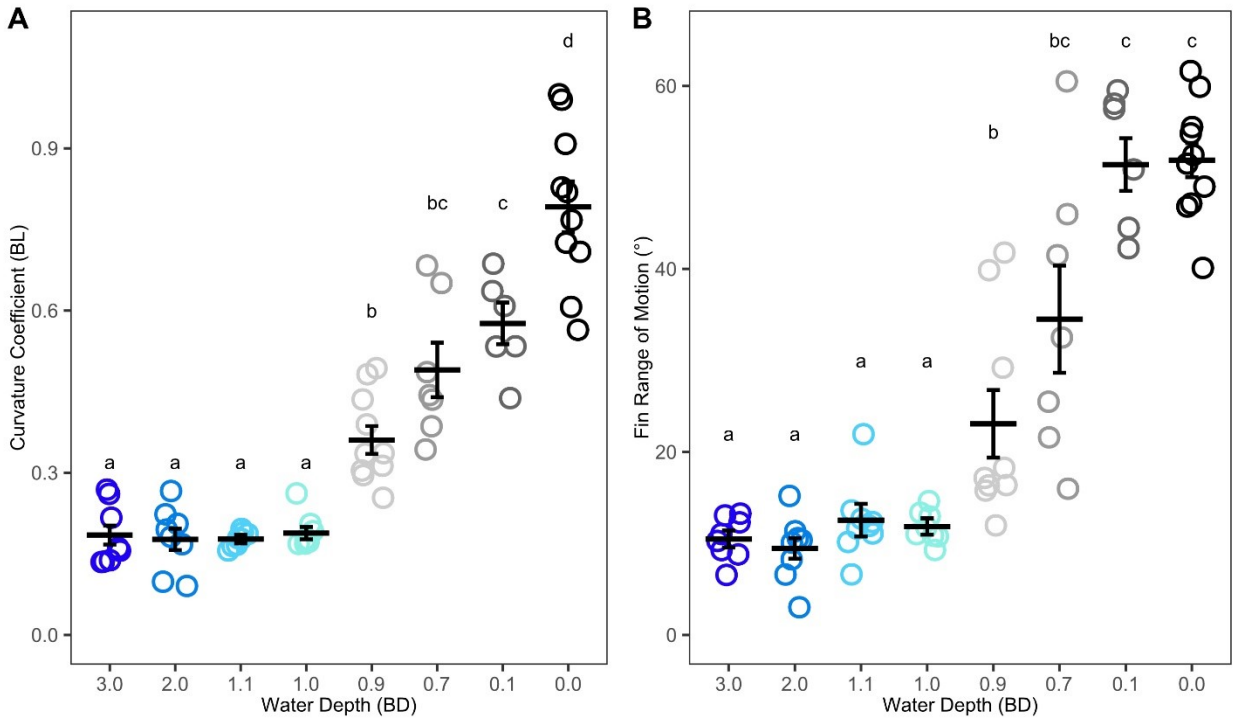

Figure 3.2. Fin and body movement changes across water depths. (A) Curvature coefficient and (B) fin range of motion show a significant increase when water depth drops to 0.9 BD (N=5 individuals). Lower-case letters denote means that are statistically similar between water depths. Individual points are the mean for each trial; thick horizontal lines and error bars are the estimated marginal mean ± s.e.m for each water depth.

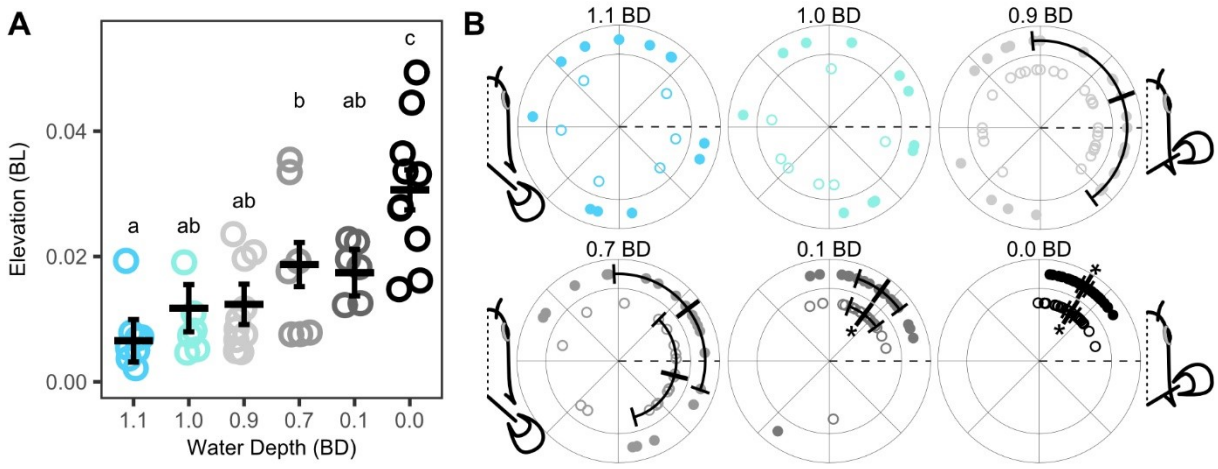

Figure 3.3. Nose elevation increases and is less variable below 0.1 BD. (A) Nose elevation magnitude and (B) timing relative to the pectoral fin stroke (N=5 individuals). Each point in (A) is a trial mean. Estimated marginal means and their s.e.m. are shown by the thick horizontal bar and error bars. Lower-case letters denote means that are statistically similar between water depths. In (B) timing of maximum elevation during swing to the right (filled circles) and left (open circles) is plotted relative to right and left fin cycles, respectively. Nose elevation timing is directional for elevations to the right in 0.9 BD and for both left and right elevation in 0.7 BD, 0.1 BD and 0.0 BD. When nose elevation timing is directional, a circular mean ± angular variance is plotted (thick bar and error bars). Nose elevation timing is less variable in 0.1 and 0.0 BD than at 0.9 BD and higher. Each point is an individual maximum elevation. 0 degrees (dotted line) is the start of fin adduction for the right and left fins; 180 degrees is the start of fin abduction for the right and left fins. Asterisks denote significantly lower angular dispersion than water depths above 0.1 BD.

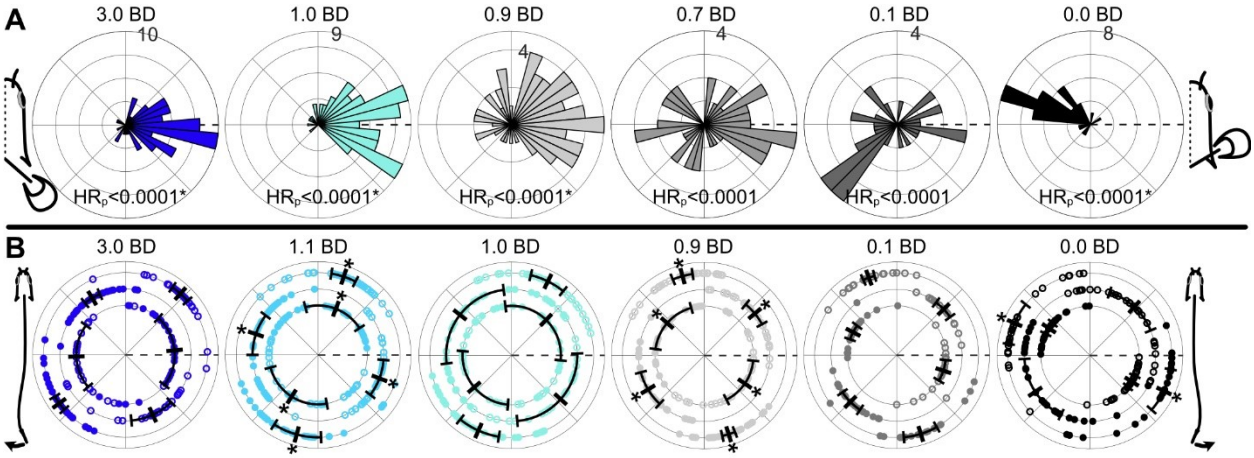

Figure 3.4. Fin and body timing changes across water depths. (A) Left pectoral fin timing; start of right fin adduction at 0 degrees – dashed line; start of right fin abduction at 180 degrees (N=5 individuals). Timing of the start of left pectoral fin adduction is binned in 10° increments and presented as histograms. The Hermans-Rasson p-value is shown at the bottom of each plot. Asterisks denote cases where the Rayleigh's test p-value is <0.05, indicating data have a unimodal distribution. (B) Left (closed circles) and right (open circles) maximum body amplitude relative to the tail stroke; start of tail swing to the right is 0 degrees – dashed line; start of tail swing to the left is at 180 degrees. Vertical bars and error bars represent the circular mean and angular variance. Data are shown for 40%, 60% and 80% body length (inner to outer ring in each plot). Asterisks denote angular means that are statistically different from the same position in the next highest water depth shown.

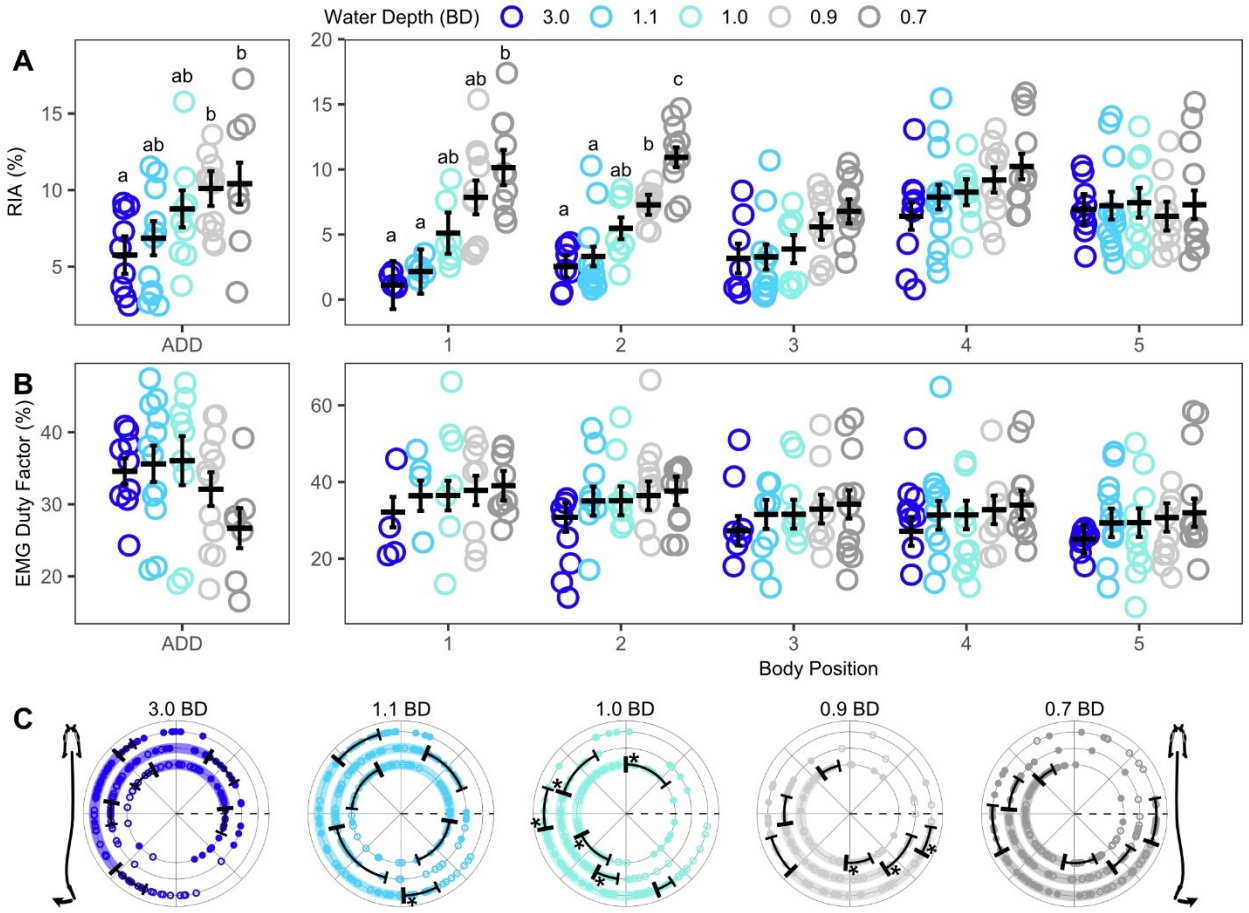

Figure 3.5. Muscle activity changes across water depth in *P. senegalus*. (A) Rectified integrated area (RIA) increases as water depth decreases for the fin adductor muscle and the anterior two positions on the body. (B) EMG duty factor shows no statistically significant changes as water depth decreases. (C) Muscle onset (closed circle) and offset (open circles) for axial red muscle. In (A) and (B) small points are trial means and the estimated marginal mean ± s.e.m. is presented as a thick horizontal line and error bars. Lower-case letters denote means that are statistically similar between water depths within a position and are only present where statistical differences occur. ADD is fin adductor muscle, 1 through 5 are body positions as defined in the text. In (C) small points are individual observations; vertical bars and error bars represent the circular mean and angular variance (angular variance only shown on one side of the mean). The portion of the cycle when each muscle is active is represented by a bar of colour between the mean onset and offset time. Data are shown for body positions 3, 4 and 5 (inner to outer ring in each plot; see text for details). N=4 individuals for all depths except 0.7 BD where N=2; see text for details. Asterisks denote angular means that are statistically different from the angular mean at the same position in the next highest water depth shown.

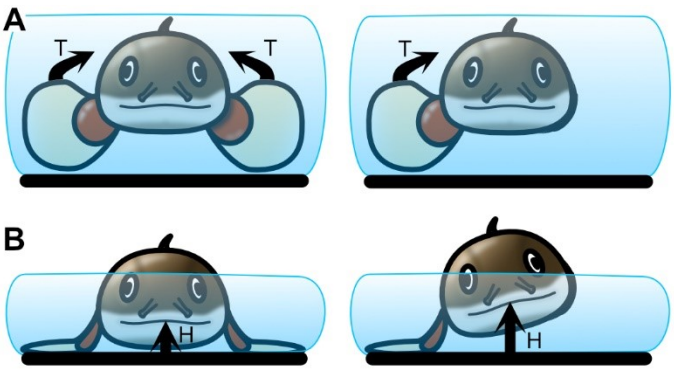

Figure 3.6. Environmental forces influence locomotor mode in *P. senegalus*. Schematic diagrams of the influence of in- and out-of-phase pectoral fin movements in (A) an aquatic environment and (B) 0.7 BD. In-phase pectoral fin movements (left panel in A and B) result in (A) balanced torque and thrust forces on the fish in an aquatic environment, (B) but are limited in their ability to lift the head off the ground in 0.7 BD. Out-of-phase pectoral fin movements result in (A) unbalanced torque and thrust forces on the fish in an aquatic environment, but (B) allows the body to roll and lift the nose of the fish higher off the ground in 0.7 BD. F_b – buoyant force, F_d – drag force, F_g – gravitational force, F_f – frictional force, T – torque, H – height.

Table 3.1. Summary of F values for linear mixed effects models.

Variable	R^2m [R^2c]	Speed	Depth	Site	Depth X Site	Depth X Speed
Locomotion Speed (BL/s)	0.230 [0.460]	-	3.85 (7, 53)*	n/a	n/a	-
Curvature Coefficient (BL)	0.852 [0.856]	-	51.66 (7, 53)*	n/a	n/a	-
Swing Distance (BL)	0.922 [0.943]	10.46 (1, 336)*	45.60 (7, 336)*	2.64 (5, 336)	9.56 (35, 336)*	-
Wave Frequency (cycles s^{-1})	0.852 [0.926]	14.38 (1, 235)*	10.32 (7, 235)*	0.14 (3, 235)	-	16.26 (7, 235)*
Fin Frequency (cycles s^{-1})	0.598 [0.605]	0.05 (1, 45)	21.56 (7, 45)*	n/a	n/a	6.61 (7, 45)*
Fin RoM (rad)	0.854 [0.863]	-	115.54 (7, 53)*	n/a	n/a	-
Nose Elevation (BL)	0.475 [0.665]	-	12.87 (5, 37)*	n/a	n/a	-
Body EMG Duty Factor (% cycle)	0.081 [0.357]	-	2.90 (4, 210)*	3.55 (4, 210)*	-	-
Pectoral Fin EMG Duty Factor (% cycle)	0.127 [0.127]	-	1.74 (4, 41)	n/a	n/a	-
Body RIA (% Max)	0.268 [0.337]	-	7.10 (4, 215)*	5.68 (4, 215)*	3.13 (16, 215)*	-
Pectoral Fin RIA (% Max)	0.318 [0.487]	5.56 (1, 40)*	4.95 (4, 40)*	n/a	n/a	-

R^2m is for the fixed effects in the model. R^2c is for the whole model. Degrees of freedom (numerator, denominator) are reported in parentheses after each F-value. Asterisk denotes $p < 0.05$. Dash indicates a term not included in the model (based on AIC criterion best fit model). "n/a" is placed in cells where there is only one site possible.

Table 3.2. Summary of pairwise comparisons within site, across water depths for each kinematic variable.

Variable	Site	Depth (BD)							
		3.0	2.0	1.1	1.0	0.9	0.7	0.1	0.0
Locomotion Speed (BL s^{-1})	-	$ab^{0.705[0.128]}$	$ab^{0.665[0.128]}$	$ab^{0.724[0.128]}$	$ab^{0.756[0.128]}$	$a^{0.952[0.120]}$	$ab^{0.850[0.133]}$	$b^{0.426[0.141]}$	$b^{0.412[0.120]}$
Curvature Coefficient (BL)	-	$a^{0.185[0.018]}$	$a^{0.177[0.020]}$	$a^{0.177[0.008]}$	$a^{0.188[0.011]}$	$b^{0.361[0.026]}$	$bc^{0.490[0.050]}$	$c^{0.576[0.039]}$	$d^{0.792[0.047]}$
Swing Distance (BL)	H	$ab^{0.147[0.025]}$	$a^{0.087[0.025]}$	$a^{0.113[0.025]}$	$a^{0.112[0.025]}$	$ab^{0.175[0.024]}$	$bc^{0.248[0.026]}$	$c^{0.334[0.028]}$	$d^{0.463[0.024]}$
	20	$a^{0.161[0.023]}$	$a^{0.106[0.023]}$	$a^{0.115[0.023]}$	$a^{0.137[0.023]}$	$a^{0.147[0.022]}$	$a^{0.170[0.024]}$	$ab^{0.199[0.026]}$	$b^{0.266[0.022]}$
	40	$ab^{0.184[0.023]}$	$a^{0.121[0.023]}$	$a^{0.122[0.023]}$	$ab^{0.141[0.023]}$	$ab^{0.182[0.022]}$	$b^{0.225[0.024]}$	$bc^{0.244[0.026]}$	$c^{0.331[0.022]}$
	60	$abc^{0.199[0.025]}$	$a^{0.136[0.025]}$	$a^{0.145[0.025]}$	$ab^{0.161[0.025]}$	$bcd^{0.255[0.024]}$	$d^{0.317[0.026]}$	$d^{0.327[0.028]}$	$cd^{0.295[0.024]}$
	80	$ab^{0.219[0.040]}$	$a^{0.162[0.040]}$	$ab^{0.193[0.040]}$	$ab^{0.206[0.040]}$	$bc^{0.354[0.036]}$	$cd^{0.451[0.041]}$	$cd^{0.531[0.044]}$	$d^{0.586[0.036]}$
	T	$a^{0.333[0.059]}$	$a^{0.273[0.059]}$	$a^{0.358[0.059]}$	$a^{0.370[0.059]}$	$ab^{0.551[0.053]}$	$b^{0.688[0.063]}$	$c^{1.109[0.074]}$	$c^{1.245[0.053]}$
Wave Frequency (cycles s^{-1})	40	$a^{3.29[0.191]}$	$abcd^{3.71[0.244]}$	$bc^{3.80[0.189]}$	$abc^{3.81[0.202]}$	$abcd^{3.79[0.217]}$	$bd^{4.03[0.189]}$	$d^{4.52[0.226]}$	$ac^{3.40[0.212]}$
	60	$a^{3.31[0.191]}$	$abcd^{3.73[0.244]}$	$bc^{3.81[0.189]}$	$abc^{3.82[0.202]}$	$abcd^{3.80[0.217]}$	$bd^{4.05[0.189]}$	$d^{4.53[0.227]}$	$ac^{3.42[0.212]}$
	80	$a^{3.34[0.191]}$	$abcd^{3.76[0.244]}$	$bc^{3.84[0.189]}$	$abc^{3.85[0.202]}$	$abcd^{3.83[0.217]}$	$bd^{4.08[0.189]}$	$d^{4.56[0.227]}$	$ac^{3.44[0.212]}$
	T	$a^{3.30[0.191]}$	$abcd^{3.72[0.244]}$	$bc^{3.80[0.189]}$	$abc^{3.81[0.202]}$	$abcd^{3.79[0.217]}$	$bd^{4.04[0.189]}$	$d^{4.52[0.226]}$	$ac^{3.40[0.212]}$
Fin Frequency (cycles s^{-1})	-	$ab^{6.20[0.320]}$	$a^{6.48[0.166]}$	$abc^{7.16[0.371]}$	$bc^{7.65[0.332]}$	$c^{8.05[0.364]}$	$abc^{7.16[0.833]}$	$abcd^{6.32[1.366]}$	$d^{2.86[0.277]}$
Fin RoM (rad)	-	$a^{0.183[0.016]}$	$a^{0.166[0.019]}$	$a^{0.193[0.019]}$	$a^{0.207[0.016]}$	$b^{0.405[0.057]}$	$bc^{0.603[0.101]}$	$c^{0.897[0.051]}$	$c^{0.906[0.032]}$
EMG Duty Factor (% cycle)	20	$a^{0.321[0.040]}$	-	$a^{0.364[0.040]}$	$a^{0.365[0.039]}$	$a^{0.378[0.039]}$	$a^{0.390[0.038]}$	-	-
	33	$a^{0.308[0.038]}$	-	$a^{0.350[0.038]}$	$a^{0.351[0.038]}$	$a^{0.364[0.037]}$	$a^{0.377[0.037]}$	-	-
	43	$a^{0.273[0.038]}$	-	$a^{0.315[0.038]}$	$a^{0.316[0.038]}$	$a^{0.329[0.038]}$	$a^{0.342[0.037]}$	-	-
	57	$a^{0.271[0.038]}$	-	$a^{0.314[0.037]}$	$a^{0.314[0.037]}$	$a^{0.328[0.037]}$	$a^{0.340[0.037]}$	-	-
	67	$a^{0.251[0.038]}$	-	$a^{0.293[0.037]}$	$a^{0.294[0.037]}$	$a^{0.307[0.037]}$	$a^{0.320[0.037]}$	-	-
	PF	$a^{0.346[0.027]}$	-	$a^{0.356[0.024]}$	$a^{0.361[0.027]}$	$a^{0.321[0.024]}$	$a^{0.267[0.031]}$	-	-
RIA (% Max)	20	$a^{1.06[1.86]}$	-	$a^{1.97[1.72]}$	$ab^{5.07[1.61]}$	$ab^{7.67[1.34]}$	$b^{10.24[1.39]}$	-	-
	33	$a^{2.69[0.91]}$	-	$a^{3.15[0.83]}$	$ab^{5.43[0.91]}$	$b^{7.10[0.85]}$	$c^{11.01[0.85]}$	-	-
	43	$a^{3.32[1.16]}$	-	$a^{3.16[1.00]}$	$a^{3.82[1.11]}$	$a^{5.38[1.03]}$	$a^{6.87[0.97]}$	-	-
	57	$a^{6.58[1.13]}$	-	$a^{7.72[1.03]}$	$a^{8.23[1.09]}$	$a^{9.01[1.06]}$	$a^{10.30[1.06]}$	-	-
	67	$a^{7.10[1.26]}$	-	$a^{7.06[1.14]}$	$a^{7.42[1.21]}$	$a^{6.23[1.17]}$	$a^{7.36[1.17]}$	-	-
	PF	$a^{5.75[1.23]}$	-	$ab^{6.86[1.13]}$	$ab^{8.78[1.21]}$	$b^{10.10[1.14]}$	$b^{10.42[1.37]}$	-	-

Pairwise comparisons are Bonferroni-corrected for multiple comparisons based on the number of comparisons within each kinematic variable. Mean values are statistically the same between depths where letters within a row are the same. Dashes indicate sites where a variable was not calculated. Site locations other than the head (H), tail (T) and adductor muscle of the pectoral fin (PF) are in % BL. Estimated marginal means are presented with their s.e.m. in square brackets as superscript.

Table 3.3. Summary of pairwise comparisons within water depth across body points for each variable.

Variable	Depth (BD)	Site					
		H	20	40	60	80	T
Swing Distance (BL)	3.0	a	a	a	a	a	a
	2.0	a	a	a	a	a	a
	1.1	a	a	a	ab	ab	b
	1.0	a	a	a	ab	ab	b
	0.9	ab	a	ab	bc	cd	d
	0.7	ab	a	ab	bc	cd	d
	0.1	a	b	ab	a	c	d
	0.0	a	b	b	b	a	c
Wave Frequency (s^{-1})	3.0	-	-	a	a	a	a
	2.0	-	-	a	a	a	a
	1.1	-	-	a	a	a	a
	1.0	-	-	a	a	a	a
	0.9	-	-	a	a	a	a
	0.7	-	-	a	a	a	a
	0.1	-	-	a	a	a	a
	0.0	-	-	a	a	a	a
		20	**33**	**43**	**57**	**67**	
EMG Duty Factor (% cycle)	3.0	a	a	a	a	a	
	1.1	a	a	a	a	a	
	1.0	a	a	a	a	a	
	0.9	a	a	a	a	a	
	0.7	a	a	a	a	a	
	3.0	a	a	a	a	a	
RIA (% Max)	1.1	abc	a	ab	c	bc	
	1.0	a	a	a	a	a	
	0.9	a	a	a	a	a	
	0.7	ab	a	b	ab	ab	

Pairwise comparisons are Bonferroni-corrected for multiple comparisons based on the number of pairs within each kinematic variable. Mean values are statistically the same when letters within a row are the same. Dashes indicate sites where a variable was not calculated. Site locations other than the head (H) and tail (T) are in % BL. Estimated marginal means and their s.e.m. can be found in Table 3.2.

Table 3.4. Summary of pairwise comparisons between angular means within each position, across water depths.

Variable	Site (% BL)	Depth (BD)							
		3.0	2.0	1.1	1.0	0.9	0.7	0.1	0.0
Maximum Left (rad)	40	$a^{0.09[0.75]}$	$a^{0.14[0.57]}$	$b^{1.20[0.63]}$	$ab^{0.80[0.90]}$	$c^{2.43[0.59]}$	$c^{3.00[0.68]}$	$c^{2.66[0.16]}$	$c^{2.56[0.13]}$
	60	$a^{2.10[0.13]}$	$a^{2.16[0.37]}$	$b^{2.82[0.32]}$	$ab^{2.48[0.79]}$	$c^{3.68[0.28]}$	$c^{4.08[0.49]}$	$c^{3.93[0.19]}$	$c^{3.72[0.53]}$
	80	$a^{3.77[0.10]}$	n/a	$b^{4.45[0.30]}$	$b^{4.30[0.26]}$	$c^{5.01[0.08]}$	$c^{5.20[0.24]}$	$c^{5.01[0.21]}$	$d^{5.84[0.28]}$
Maximum Right (rad)	40	$ab^{3.18[0.67]}$	$a^{2.99[0.24]}$	$c^{4.14[0.75]}$	$bc^{3.85[0.79]}$	$d^{5.68[0.50]}$	$d^{6.16[0.66]}$	$d^{5.96[0.22]}$	$d^{5.73[0.13]}$
	60	$a^{5.14[0.34]}$	$a^{5.04[0.52]}$	$b^{5.92[0.29]}$	$ab^{5.55[0.76]}$	$c^{0.69[0.20]}$	$c^{1.06[0.84]}$	$c^{0.77[0.16]}$	$c^{0.83[0.44]}$
	80	$a^{0.84[0.12]}$	$a^{0.66[0.51]}$	$b^{1.25[0.16]}$	$ab^{1.11[0.22]}$	$c^{1.84[0.13]}$	$c^{2.05[0.64]}$	$c^{1.92[0.07]}$	$d^{2.74[0.27]}$
EMG onset (rad)	43	$a^{0.10[0.51]}$	-	$a^{6.13[1.07]}$	$b^{1.57[0.95]}$	$bc^{2.18[0.39]}$	$c^{2.37[0.37]}$	-	-
	57	$a^{1.05[0.56]}$	-	$a^{1.18[0.80]}$	$b^{2.82[0.77]}$	$b^{3.30[0.42]}$	$b^{3.07[0.49]}$	-	-
	67	$a^{2.35[0.22]}$	-	$a^{2.44[0.60]}$	$b^{3.33[0.49]}$	$b^{3.91[0.48]}$	$b^{3.63[0.59]}$	-	-
EMG offset (rad)	43	$a^{2.05[0.52]}$	-	$a^{2.03[1.01]}$	$b^{3.58[0.77]}$	$c^{4.64[0.52]}$	$c^{4.49[0.64]}$	-	-
	57	$a^{2.97[0.34]}$	-	$a^{3.35[1.13]}$	$b^{4.24[0.35]}$	$c^{5.28[0.66]}$	$c^{5.33[0.42]}$	-	-
	67	$a^{3.88[0.44]}$	-	$b^{4.75[0.43]}$	$b^{5.09[0.21]}$	$c^{6.08[0.34]}$	$c^{5.93[0.55]}$	-	-

Pairwise comparisons are Bonferroni-corrected for multiple comparisons based on the number of comparisons within each kinematic variable. Mean values are statistically the same between depths where letters within a row are the same. Dashes indicate sites where a variable was not calculated. Angular means with their angular variance (square brackets) are presented in superscript. 'n/a' indicates where data were not directional (Rayleigh's test p-value < 0.05).

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3.8 Supplementary Material

Additional supplementary material (R code for linear statistics, Matlab code for polar statistics and datasets) can be found here. Movies can be accessed as clickable YouTube links below.

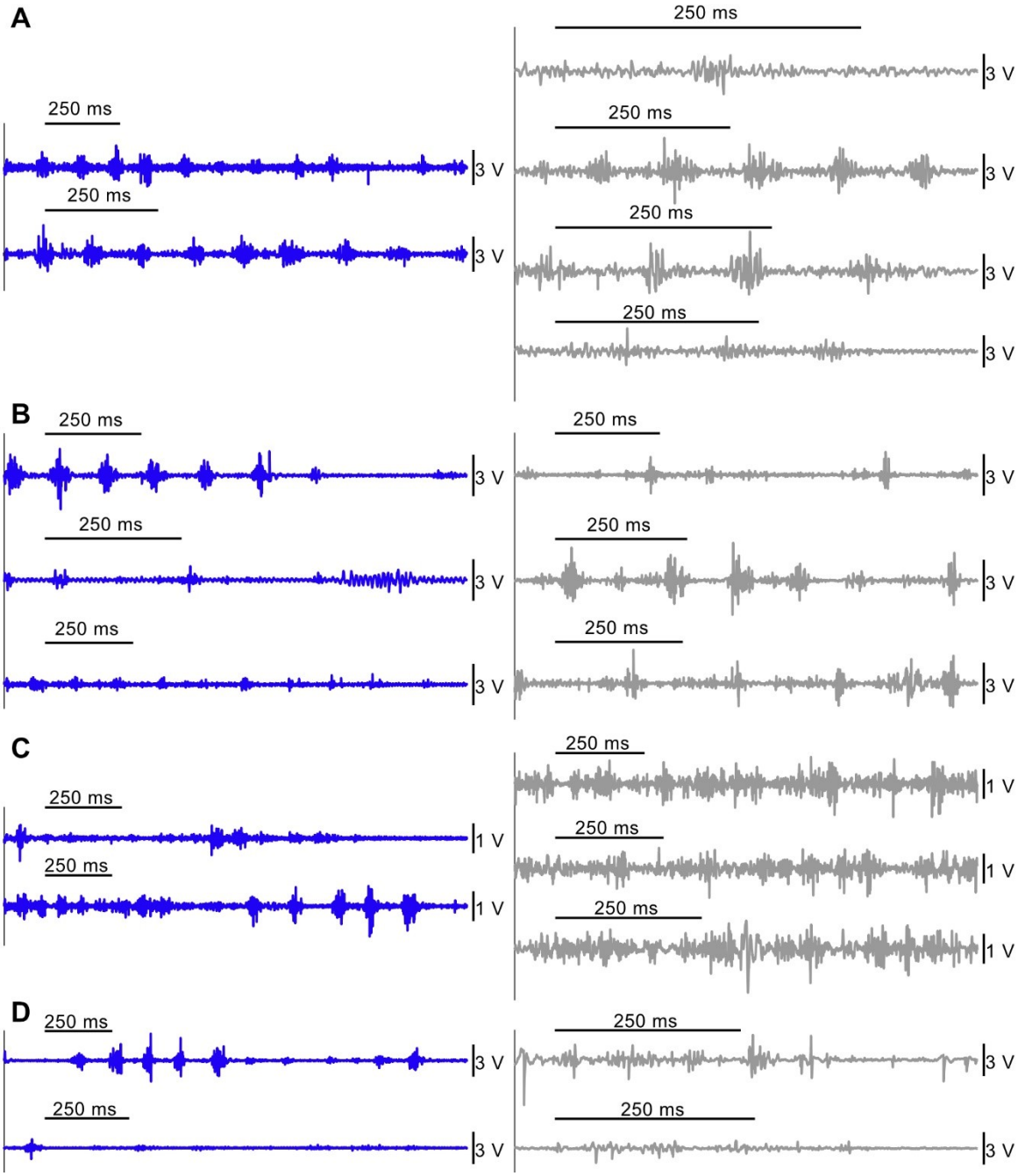

Figure S3.1. Muscle activity traces from the adductor of the pectoral fin from 3.0 BD and 0.7 BD for each fish in the EMG set. Each of (A) through (D) is an individual fish. Blue traces in the left column are 3.0 BD trials. Grey traces in the right column are 0.7 BD trials. Each trace is a trial. (A) and (B) were quantified with onsets and offsets in 0.7 BD and therefore included in the duty factor and RIA datasets. (C) and (D) do not show consistent, discrete bursts in 0.7 BD, were not quantified using onsets and offsets at this depth, and thus were not included in the duty factor and RIA datasets at this depth.

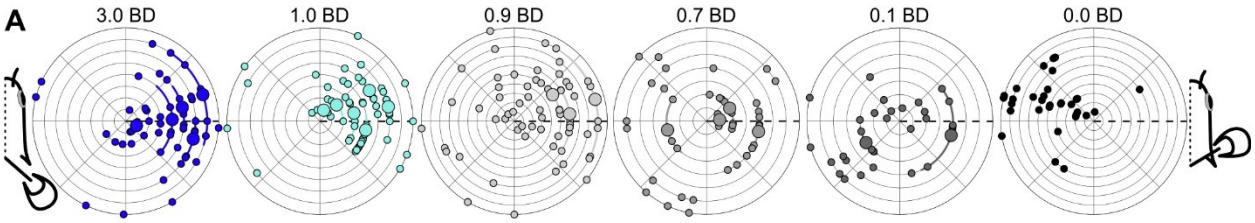

Figure S3.2. Pectoral fin timing changes across water depths. Left pectoral fin timing; start of right fin adduction at 0 degrees – dashed line; start of right fin abduction at 180 degrees (N=5 individuals). Each ring is a trial; small points are individual observations of the start of left fin adduction, and large points are angular means for each trial. Angular means and their 95% confidence interval (lines) are presented only when data is directional (Rayleigh's test p-value < 0.05) within a trial.

Table S3.1. Linear mixed effects model forms.

Dependant Variable	Fixed Effects	Random Effects	Variance Structure
Locomotion Speed	Depth	~1|Fish	n/a
Curvature Coefficient	Depth	~1|Fish	~1|Depth
Swing Distance	Depth*Site	~1|Fish	~1|Site
Wave Frequency	Speed*Depth+Site	~1|Fish	~1|Depth
Fin Frequency	Speed*Depth	~1|Fish	~1|Depth
Fin RoM	Depth	~1|Fish	~1|Depth
Nose Elevation	Depth	~1|Fish	n/a
Body EMG Duty Factor	Depth+Site	~1|Fish	n/a
Pectoral Fin EMG Duty Factor	Depth	~1|Fish	~1|Depth
Body RIA	Depth*Site	~1|Fish	~1|Site
Pectoral Fin RIA	Speed+Depth	~1|Fish	n/a

Depth is water depth, Site is position on the body, Speed is locomotion speed, Fish is fish identity. A plus sign indicates no interaction between factors; an asterisk indicates an interaction between factors. ~1| before a random effect indicates that the intercept can vary randomly. ~1| in the variance structure indicates that the variance estimate for that effect is allowed to vary. "n/a" is placed where no variance structure was needed.

Movie 3.1. Swimming (top panel) and walking (bottom panel) from two different fish (kinematics set) at a similar speed. Water depth is shown in the top left corner of each panel. Scale bar is 20 mm. BD – body depths, BL – fish total body length.

Movie 3.2. Locomotion (same fish at a similar speed; kinematics set) in intermediate water depths showing changes in body movement. Water depth is shown in the top left corner of each panel. Scale bar is 20 mm. BD – body depths, BL – fish total body length.

Movie 3.3. Locomotion (different fish at a similar speed; kinematics set) in 0.7 BD with discrete differences in fin coordination between trials. Water depth is shown in the top left corner of each panel. Scale bar is 20 mm. BD – body depths, BL – fish total body length.

CHAPTER 4: SEARCHING FOR A MESENCEPHALIC LOCOMOTOR REGION IN *POLYPTERUS SENEGALUS*

Chapter Notes

This chapter is presented only as part of this thesis.

Author contributions

K.L. and E.M.S. shared project conceptualization, experimental and methods design, and writing of the manuscript. K.L. collected and analyzed the data. K.L. wrote the analysis code.

Acknowledgements

We thank John Lewis, Tuan Bui, and Dimitri Ryczko for helpful discussions about experimental protocol and setup. Additionally, we thank all members of our Human Frontiers Science Program Grant team.

4.1 Abstract

The functionally conserved mesencephalic locomotor region (MLR): initiates locomotion, controls the frequency of locomotor movements, and elicits locomotor transitions across vertebrates from lamprey to primates (reviewed in Grillner and El Manira 2020). The MLR is found in two regions of the midbrain: the pedunculopontine nucleus and the laterodorsal tegmental nucleus (LDT). Because of the ubiquitous nature of the MLR and its role in the control of both locomotor transitions and locomotion speed, we sought to identify a putative MLR in the amphibious fish *Polypterus senegalus* and determine its function in this species. Investigating the role of the MLR in *P. senegalus* is particularly interesting because of the unique set of locomotor transitions that occur from low- (powered by pectoral fins only) to mid- (powered by pectoral fins and body undulation) to high-speed (powered only by body undulation) swimming, and (when on flat terrestrial terrain) during walking. To test for the presence of an MLR we used immunohistochemistry in the brain and spinal cord to confirm previous work that found choline acetyltransferase positive neurons and neuronal tracts in the LDT and spinal cord of *P. senegalus*. We then determined whether electrical stimulation in this region generated repeated and rhythmic body and fin movements. We successfully repeated previous immunohistochemistry findings, but only one of 33 preparations responded to stimulation of the putative MLR with repeatable and rhythmic anterior-posterior waves of lateral undulation. In this one preparation stimulation frequency was positively correlated with body undulation frequency, but the amplitude envelope was markedly different from that of normal swimming. These results suggest that the MLR in *P. senegalus* likely controls the frequency of axial undulation, but that it does not appear to control locomotor transitions or pectoral fin movements. Further investigations with this type of preparation may be fruitful for clarifying the role of the MLR in *P. senegalus*.

4.2 Introduction

4.2.1 The mesencephalic locomotor region: position and function

The mesencephalic locomotor region (MLR) is a functionally defined region of the midbrain that was originally found in cats (Shik et al., 1966; Shik et al., 1969), but has since been identified in a variety of vertebrates from lamprey to primates (reviewed in Grillner and El Manira, 2020). Electrical stimulation of this well conserved structure initiates locomotion and controls locomotor speed and/or locomotor transitions, depending on the species. Based on optogentic studies of mouse neurocircuits and behaviour, current evidence suggests that the MLR lies in the pedunculopontine nucleus (PPN; Caggiano et al., 2018; Josset et al., 2018). However, studies in lamprey (Sirota et al., 2000) and salamander (Cabelguen et al., 2003) determined that populations of cholinergic neurons in both the PPN and laterodorsal tegmental nucleus (LDT), nearby midbrain structures, likely constitute the MLR in these species.

In the lamprey, an anguilliform swimmer, electrical stimulation of the MLR initiates and controls the speed of swimming movements (Sirota et al., 2000). In the amphibious salamander, electrical stimulation of the MLR initiates locomotion, controls the speed of movements, and facilitates a transition from walking at low levels of stimulation, to swimming at high levels of stimulation (Cabelguen et al., 2003). A central pattern generator (CPG)-based robotic model of the salamander neural control system suggests that this locomotor transition in salamanders could be accomplished by a single neural circuit with limb "neural" thresholds that saturate at higher intensity drive (i.e. signals from the modelled MLR). This higher drive generates tonic contraction of the limbs against the body (as is observed in the live animal during swimming) (Delvolvé et al., 1997; Ijspeert et al., 2007). This robotic model further suggests that effective amphibious locomotion can be generated by adding limb CPGs on top of a lamprey-like spinal CPG network (Ijspeert et al.,

2007). This begs the question: are the neuromotor circuits of other amphibious animals with appendages organized in a similar manner?

4.2.2 Locomotor transitions in Polypterus senegalus

Polypterus senegalus is a predominantly aquatic actinopterygian that is capable of terrestrial locomotion (Du et al., 2016; Standen et al., 2014; Standen et al., 2016). Low-speed swimming in this species is powered by in-phase pectoral fin oscillation (Standen et al., 2014; K.L. pers. obs.; Fig. 4.1). Body and tail oscillations are recruited as swimming speed progressively increases (K.L. pers. obs. Fig. 1). At their highest swimming speeds, the pectoral fins of *P. senegalus* are tucked and the body and caudal fin provide all propulsion (K.L. pers obs.; Fig. 4.1). Terrestrial locomotion is plastic, depending on substrate (Standen et al., 2016), but over flat ground this species walks by planting its pectoral fins on the ground and vaulting the nose and body over the planted fin (Standen et al., 2014; Standen et al., 2016; Fig. 4.1). In this way, rather than a bifurcation in locomotor mode between swimming and walking (as seen in salamanders), *P. senegalus* have (at least) three locomotor transitions: (1) from pectoral fin swimming to body-caudal and pectoral fin swimming as swimming speed increases, (2) from body-caudal and pectoral fin swimming to body-caudal swimming as swimming speed increases further and (3) from swimming to walking (Fig. 4.1). Unlike the salamander, these behaviours are not confined to discrete locomotor frequency ranges. In the salamander, swimming cycle frequencies are between 1.5 and 3.5 Hz, while walking cycle frequencies are between 0.5 and 1.25 Hz with a distinct gap between the frequency ranges for these locomotor modes (Delvolvé et al., 1997). In *P. senegalus*, on the other hand, swimming and walking can occupy similar body frequency ranges (though note pectoral fin frequency is lower during walking); however, actual locomotion speed may differ because of the dramatic differences in physical environment between water and land (Chapter 3). However, recent evidence (Chapter 3) suggests that there are similarities in the axial motor control pattern during swimming and walking and

kinematic differences are the result of physical constraints of the terrestrial environment. Thus, the main difference between swimming and walking in this species appears to be a change from in-phase to out-of-phase pectoral fin movements based on the environmental conditions. That the body changes continuously and the pectoral fins transition discretely from swimming to walking may suggest relatively weak neural coupling between the pectoral fins and body. If coupling between these primary locomotor structures is weak, it may be more appropriate to conceptualize the locomotor transitions above as separate transitions, one for the body and one for the fins, potentially with different triggers (Fig. 4.1). Body movements vary continuously from no to high lateral amplitude and pectoral fin movements may go from in-phase or absent in aquatic environments to out-of-phase when the pectoral fins interact with the substrate below.

Because of the seemingly ubiquitous presence of an MLR across vertebrates (indeed the presence of an MLR has also been demonstrated in fish; Kashin et al., 1974), and because of the unique locomotor transitions observed in *P. senegalus*, the present investigation aims to answer two questions: (1) does *P. senegalus* have an MLR and (2) if present, what is the function of the MLR for locomotor control of the body and pectoral fins. To answer these questions, we use a decerebrate, semi-intact preparation to first test whether there is a location in the brain that elicits rhythmic locomotion and second to examine the effect of stimulation frequency and stimulation intensity on these rhythmic movements.

4.3 Methods

The methods for the decerebrate preparation and electrical brain stimulation presented here are largely based on previous investigations of the lamprey (Sirota et al., 2000) and salamander (Cabelguen et al., 2003), since these are the most detailed methodologies with the closest phylogenetic relationship to *P. senegalus*.

4.3.1 Animals

Polypterus senegalus (n = 33, total length = 102.4 ± 1.1 mm, mass = 5.29 ± 0.16 g) were obtained from several pet trade suppliers (Mirdo Importations Canada, Montreal, QC, CA; Aquality Tropical Fish Wholesale, Mississauga, ON, CA). Fish were housed in individual, recirculating tanks at 25-27 °C on a 12/12 light/dark cycle and were fed at least twice a week with a mix of beef heart and sinking fish pellets. All protocols and procedures were performed according to the University of Ottawa Animal Care protocol BL-2388.

4.3.2 Experimental Procedure

Prior to surgery, fish were deeply anaesthetized in buffered tricane methanesulfonate (MS222; 250 mg/600 mL; Syndel Laboratories Ltd., Nanaimo, BC, CA) until breathing stopped and the fish was unresponsive to touch. Fish were then removed from anaesthetic and a decerebration was performed by removing the top of the brain case, making a transverse cut between the pallium and optic tectum, and removing the anterior piece of brain tissue. This left the mid- and hindbrain intact and attached to the spinal cord but removed the main centers of higher sensory processing (Fig. 4.2). Throughout this surgical procedure, the brain was kept moist by hand with room temperature (22 – 23 °C) artificial cerebrospinal fluid (aCSF) of the following composition (in M): 0.134 NaCl, 0.0029 KCl, 0.0021 $CaCl_2$, 0.0012 $MgCl_2$, 0.01 Hepes, 0.01 Glucose. After surgery was completed (< 10 minutes; generally ~5 minutes; Fig. 4.3), the preparation was moved to the stimulation chamber, positioned such that the partition was just posterior to the opercular flaps (leaving the opercula free to open in the anterior chamber and the pectoral fins free to move in the posterior chamber) and the head was pinned through the brain case to a Sylgard (Sylgard™ 184 Silicone Elastomer Kit; The Dow Chemical Company, Midland, MI, USA) wedge to keep the head stable (Fig. 4.2). Once the fish was securely positioned, a small tube was inserted into the mouth to perfuse the gills with fresh, oxygenated fish housing water (Fig. 4.2). A second tube was affixed just

above the brain to keep the brain irrigated with fresh, oxygenated aCSF (Fig. 4.2). Because of the nature of this setup, aCSF and fish housing water mix in the anterior chamber (and aCSF potentially flows over the gills), but the gills were continuously irrigated with fish housing water and the excess mixture was continuously removed to minimize any effect of mixing on the preparation. The posterior chamber was filled with fish water (Fig 4.2). The process of moving the animal into the chamber generally took less than 10 minutes. The preparation was then observed until the anaesthesia wore off before stimulation trials commenced. Usually the anaesthesia took 30 minutes to wear off, but in some preparations less time was required. The level of anesthesia was assessed by looking for spontaneous swimming movements or a response to tail pinching and assessing body rigor.

After the anesthesia wore off, stimulation of the brain commenced. A return electrode (silver wire) was placed in tissue along the inner wall of the brain case (Fig. 4.2) and a Parylene coated, Tungsten stimulating electrode (0.005" wide, 3" long, 2 MΩ resistance; A-M Systems, Sequim, WA, USA) was positioned near the hypothesized location of the MLR (Fig. 4.2). In the salamander, successful activation of the MLR was achieved by stimulation of the LDT. Previous choline acetyltransferase (ChAT) immunostaining identified the location of the LDT in *P. senegalus* to be along the dorsal edge of the hindbrain, in line with the anterior edge of the cerebellum (Fig. 4.2; López et al., 2013). This location in the brain corresponds to the superficial landmark of the cleft between the optic tectum and cerebellum. Because of the angle on the wedge of Sylgard to which the brain case was affixed, the stimulating electrode was first positioned just anterior to this cleft in hopes of reaching the LDT. Penetrations in this region were performed in 5-10 μm steps with a stimulation of varying intensity performed at each step. Stimulation was performed with a constant current isolated pulse stimulator (Model 2100; A-M Systems). Generally, stimulation was set at 5-10 μA (as in Cabeguen et al., 2003; Sirota et al., 2000), but this amplitude was increased or decreased to

best match the state of the preparation. Pulse duration was 2 msec and pulse frequency was between 5 and 15 Hz. Penetrations were performed until stimulation elicited repeatable rhythmic locomotor movements or until the preparation was no longer viable. Searches were generally concentrated in the hypothesized region of interest, but in a few individuals were performed across the intact brain region. When repeatable locomotor movements were elicited by electrical stimulation, the frequency and amplitude of stimulation was systematically varied until the preparation was no longer viable. All stimulations were ≤ 20 s. Search stimulation duration was 10 s. When a stimulation position elicited repeated rhythmic locomotor movements, two minutes of rest was permitted between test stimulations. Successful trials were filmed for kinematics using either a GoPro Hero 8 (125 frames per second) or an iPhone SE (60 frames per second) to characterize movement under different stimulation parameters.

Following some (n=5) trials, the brain was burned by 30 sec of constant, high current stimulation to try and localize the position of the stimulating electrode. For all trials, fish were euthanized by a prolonged duration in the anesthetic MS222 following the end of electrical stimulation. Mass and total length were recorded. For some trials, the intact brain region was removed and placed in formalin for staining and/or cryosectioning.

4.3.3 Cryosectioning, Immunostaining and Microscopy

When a brain was to be sectioned and stained, the intact brain region was placed into 10% formalin (Fisher Scientific, Waltham, MA, US) at room temperature for two hours. It was then moved to 30% sucrose in 10x phosphate buffered solution (PBS) and held at 4 °C overnight or until the tissue sank. The intact brain tissue was then embedded into optimal cutting temperature compound (Fisher Healthcare, Houston, TX, US) and frozen in acetone chilled over dry ice. The frozen tissue was kept at -80 °C until cryosectioning. The tissue was then sectioned into 20-40 μm

sections on a cryostat and mounted on slides. These sections were either imaged immediately or continued with anti-ChAT staining as follows.

For immunostaining, sections were rinsed twice in 10x PBS. Sections were then incubated with goat anti-ChAT (AB144P; Sigma-Aldrich Canada, Oakville, ON, CA) at a concentration of 1:100 in 10x phosphate buffered solution containing 0.5% Triton-X (Sigma-Aldrich Canada), 15% normal rabbit serum and 2% bovine serum albumin for 48 hours at 4 °C. Sections were then rinsed three times in 10x phosphate buffered solution (10 minutes per rinse) and incubated for 60 minutes at room temperature with AlexaFlour 488 rabbit anti-goat secondary antibody (1:500 in 10x PBS; Fischer Scientific). We then mounted coverslips above the sections with DPX (Sigma-Aldrich) and imaged the slides on a fluorescent microscope (Zeiss, Oberkochen, BW, DE).

4.3.4 Kinematic Analysis

Videos were digitized using the Matlab software DLTdv8 (Hedrick, 2008) to obtain ten points along the midline of the body of the fish posterior to the chamber partition (just anterior to the pectoral fins). These ten points were then splined using custom Matlab code to determine 101 equidistant points along the fish body midline. These midlines were used to reconstruct the amplitude envelope for bouts of stimulated swimming and to calculate tail amplitude and frequency. Amplitude envelopes were plotted in Matlab. Tail amplitude and frequency were plotted using the tidyverse and cowplot in RStudio.

4.4 Results

Trials were carried out with 33 individual *P. senegalus* with the aim of identifying the MLR in this species. Of these trials, 6 of the preparations were not viable after surgery and so were not stimulated, 21 were viable but electrical stimulation did not elicit any movements, 4 were viable and electrical stimulation elicited random or arhythmic movements, 1 was viable, stimulation elicited rhythmic movements, but this response was not repeatable, and 1 was viable, electrical stimulation

elicited rhythmic movements and this response was repeatable. Surgery length and the time between the end of surgery and getting the animal into the preparation did not appear to affect the likelihood of preparation viability, as assessed by the time from the end of surgery to first response to touch and the total time the preparation remained viable (Fig. 4.3). However, getting the animal into the preparation in less than ten minutes does appear to be key to preparation success (Fig. 4.3). While I was unable to identify any electrolytic lesions generated following trials, we did successfully identify ChAT positive cells in the region of the LDT and in the rostral spinal cord as was observed in previous studies (Fig. 4.4; López et al., 2013).

Because only one preparation displayed repeatable, rhythmic responses to electrical stimulation, I focus on describing this preparation's responses to different frequency and amplitude stimulation and assess whether there is evidence for an MLR based on this one preparation. Stimulation of the putative MLR in this preparation elicited a travelling wave of lateral undulation along the body (Fig. 4.5) and the frequency of this undulation appears to be positively correlated with stimulation frequency (Fig. 4.5, 4.6). This preparation did not display any correlation between tail amplitude and stimulation frequency (Fig. 4.5, 4.6) and the amplitude envelope and movement frequency at all stimulation frequencies was markedly different from swimming in an intact animal (Fig. 4.5). Notably, electrical stimulation never elicited regular pectoral fin movements of any kind.

4.5 Discussion

While most of the preparations showed no rhythmic, repeatable response to stimulation of the putative MLR, I was able to elicit repeatable and rhythmic axial movements in one preparation and to confirm ChAT-positive cells exist near the LDT, a brain region where MLR neurons have been found in other species.

4.5.1 Evidence for the putative MLR in P. senegalus

While the recent evidence in mice suggests that the MLR lies inside the PPN, the MLR of lamprey and salamanders appears to be a collection of cholinergic cells in the LDT and PPN (Cabelguen et al., 2003; Sirota et al., 2000). Since the PPN and LDT are found in similar anatomical locations in other species, I aimed to achieve electrical stimulation of a population of cholinergic neurons identified in the LDT of *P. senegalus* in a previous study (López et al., 2003) and confirmed by our immunohistochemistry tests (Fig. 4.4). Thus, my penetration locations were centered around the border between the optic tectum and cerebellum, aiming to reach the LDT located directly below this border in the hindbrain. Most stimulations in this region were unsuccessful, however, one preparation with the electrode placed in this region at a penetration depth of one to one and a half millimeters elicited repeated rhythmic movements (Fig. 4.5, 4.6). Further, like previous investigations in the lamprey and salamander (Cabelguen et al., 2003; Sirota et al., 2000), this preparation demonstrated a positive relationship between stimulation frequency and tailbeat frequency (Fig. 4.5, 4.6). While by no means conclusive, this successful preparation does suggest that there is a putative MLR in *P. senegalus* and that this MLR controls the frequency of repeated anterior-posterior waves of axial undulation. While this is a first piece of evidence, the marked differences in amplitude envelope, limited repeatability of the preparation success, and low undulation frequency require further investigation before a confident conclusion can be reached.

The frequency of undulation achieved by MLR stimulation in both lamprey and salamander is like that achieved by free swimming animals (Cabelguen et al., 2003; Ijspeert et al., 2007; Sirota et al., 2000; Wallén and Williams, 1984). In our *P. senegalus* preparation, the frequency of axial undulation achieved was almost half the lowest frequencies observed during routine swimming in intact animals (Fig. 4.5; pers. obs. K.L.). Below about 2 Hz tail movements, *P. senegalus* appear to shift to a pectoral fin only swimming gait, but all lateral undulation that was achieved by electrical

stimulation of the putative MLR was confined to being lower than 2 Hz. Whether this is an artifact of an inideal preparation or if the parameters of electrical stimulation were not appropriate to achieve normal swimming behaviour remains to be investigated further. Even with the one preparation what was successful, I was only able to run 15 trials, 10 of which exhibited regular axial undulations. Given more time with a viable preparation, the state space of stimulation amplitude and frequency could be more thoroughly explored and may better elucidate the influence of the MLR on locomotor behaviour in this species. It is also worth noting that MLR stimulation experiments in salamander and lamprey achieved regular swimming movements with stimulation amplitudes between 0 and 3 μA (Sirota et al., 2000; Cabelguen et al., 2003), while my one successful preparation was only responsive to a stimulation of > 16 μA. This may be indicative of a preparation that was not under ideal conditions. Alternatively, it may be that *P. senegalus* require higher levels of stimulation than the other species, or that an MLR independent pathway is responsible for initiating swimming. Further investigation with successful preparations could find a setup that allows lower levels of stimulation to elicit regular locomotor movements.

4.5.2 Polyperus senegalus gait changes

The evidence we have thus far suggests that the putative MLR controls the frequency of swimming movements (Fig. 4.5, 4.6) but does not mediate the transition from swimming to walking. While this result is incredibly preliminary, it is interesting to speculate how this lines up with other evidence for the nature of locomotor transitions in *P. senegalus*. Since at no point in any of the trials I conducted with these semi-intact preparations did I observe pectoral fin movements, this may suggest that the MLR influences the axial swimming movement frequency, but there is likely another brain region responsible for controlling pectoral fin movements in *P. senegalus*. This prediction accords with evidence presented in Chapter 3 that the transition from water to land in *P. senegalus* relies on differential modulation of axial and pectoral fin movements, suggesting a weak neural

coupling between pectoral fin and body. If different brain centers control axial and pectoral fin movements, coordination between pectoral fin and body may be reliant upon sensory synchronization. This could explain why the pectoral fin switches from synchronous movements to asynchronous movements only when the fin rays contact a substrate below (Chapter 3). Loading the pectoral fin in this way may generate sensory feedback that elicits the switch in fin coordination and synchronizes the pectoral fin and body movements (Standen et al., 2014; Standen et al., 2016; Chapter 3). This synchronization could occur if, through neuromodulation, sensory feedback increases the strength of the coupling between the body and pectoral fins.

The results presented here are in line with the hypothesis that local sensory feedback to body CPGs is required for the changes in body kinematics across the transition from swimming (low amplitude body undulation) to walking (high amplitude body undulation). The current understanding is that higher-order sensory feedback is processed in the cortex/pallium and passed to the MLR (Grillner and El Manira, 2020). Changes in integrated sensory information could therefore result in changes to the input MLR signal strength or frequency. Therefore, if integrated sensory information generated the transition from swimming to walking, a relationship between stimulation amplitude and/or frequency (which could replicate changes in integrated sensory input to the MLR) and body undulation amplitude would be expected. In contrast, the MLR stimulation results suggest that there is no such relationship since we observed no trend in body undulation amplitude in the successful preparation. The hypothesis that body kinematic changes across the transition from swimming to walking is generated by local sensory feedback directly to body CPGs could be tested by running similar stimulation experiments and introducing a substrate that contacts the body of the fish in the posterior chamber.

4.6 Conclusion

This series of investigations has confirmed that ChAT-positive cells are in the region of the LDT and suggest that anterior-posterior axial undulations can be repeatably generated by electrical stimulation of the midbrain in *P. senegalus*. This is preliminary evidence of a putative MLR in *P. senegalus* and suggests that the MLR controls the frequency of axial swimming movements but does not elicit locomotor transitions or control pectoral fin movements. Pectoral fin movements may therefore be controlled by another brain region and/or local sensory signals.

4.7 Figures

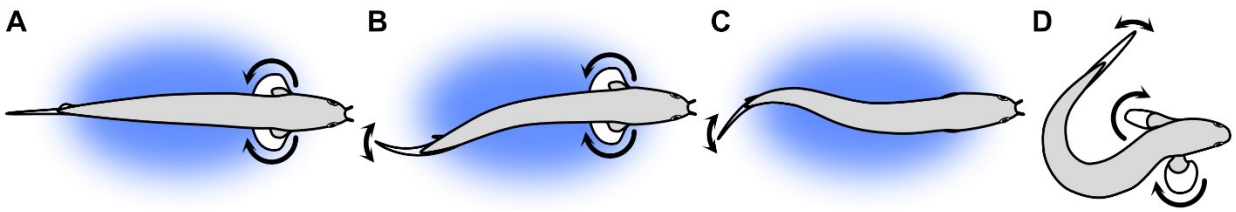

Fig. 4.1. Aquatic and terrestrial locomotor transitions of *Polypterus senegalus*. At low swimming speeds (A) *P. senegalus* use synchronous pectoral fin oscillations to power locomotion. As swimming speed increases (B) the body and tail are progressively recruited and pectoral fin frequency increases. At their fastest swimming speeds (C) *P. senegalus* swim using only body and tail undulations. When on smooth terrestrial substrates (D) *P. senegalus* plant their pectoral fins asynchronously and vault themselves over the stiffened fins using large body undulations. Black arrows denote body part motion. Pectoral fin arrow direction denotes fin synchronicity. Blue background indicates the behaviour occurs in water.

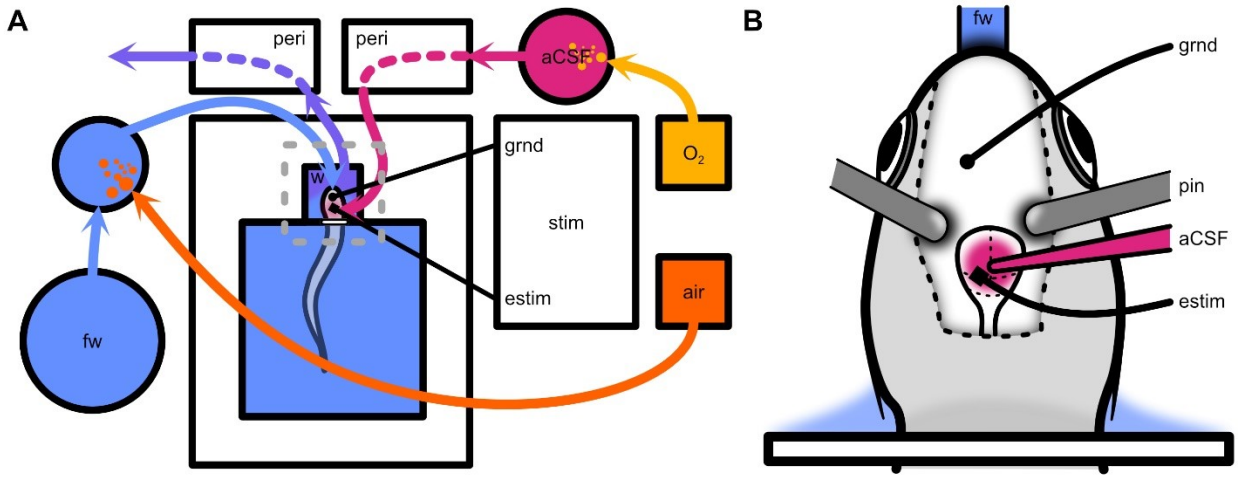

Fig. 4.2. Schematic of semi-intact preparation experimental setup. (A) Following surgery, fish were affixed in a recording chamber with the head and body separated. The body was submerged in fish housing water (fw - blue), and fish water (bubbled with air - orange) was passed over the gills. The brain was continuously wetted with artificial cerebrospinal fluid (aCSF- pink; bubbled with oxygen - yellow) supplied by a peristaltic pump (peri). Excess fw and aCSF was pumped out with a second peristaltic pump. (B) is a closeup on the area in a grey dotted box in (A). The head was pinned to Sylgard through the brain case and a ground electrode (grnd) was placed in tissue anterior to the intact section of brain. Penetrations in 25 μm steps were performed near the suture between optic tectum and cerebellum until the repeatable rhythmic behaviour was elicited by stimulation. estim – stimulating electrode; stim – electrical stimulator; w – waste liquid (combination of aCSF and fw - purple).

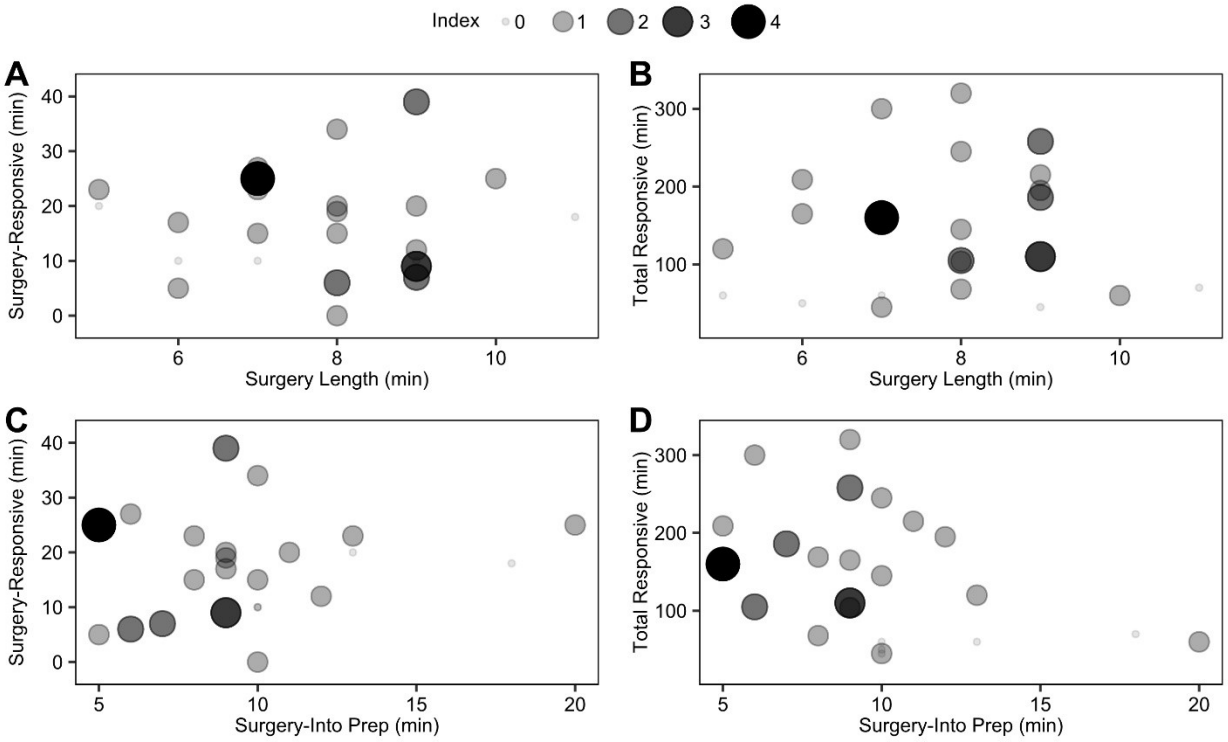

Fig. 4.3. Summary metrics for semi-intact preparations. Indicators of preparation health suggest that any level of success depends on a short elapsed time from the end of surgery to getting the animal into the preparation (Surgery-Into Prep). (A, C) Surgery-Responsive is the time that elapsed from the end of surgery to the first response to touch and (B, D) Total Responsive is the total time the preparation remained responsive to touch. Index is a categorical variable describing the success of the experiment for that preparation. 0 – stimulation not started (poor preparation health); 1 – stimulation started but no response to stimulation; 2 – stimulation started, non-repeatable movements occurred either after stimulation or spontaneously; 3 – stimulation started, repeatable movement following stimulation; 4 – stimulation started, repeatable movement and demonstrable effect of stimulation frequency on kinematics.

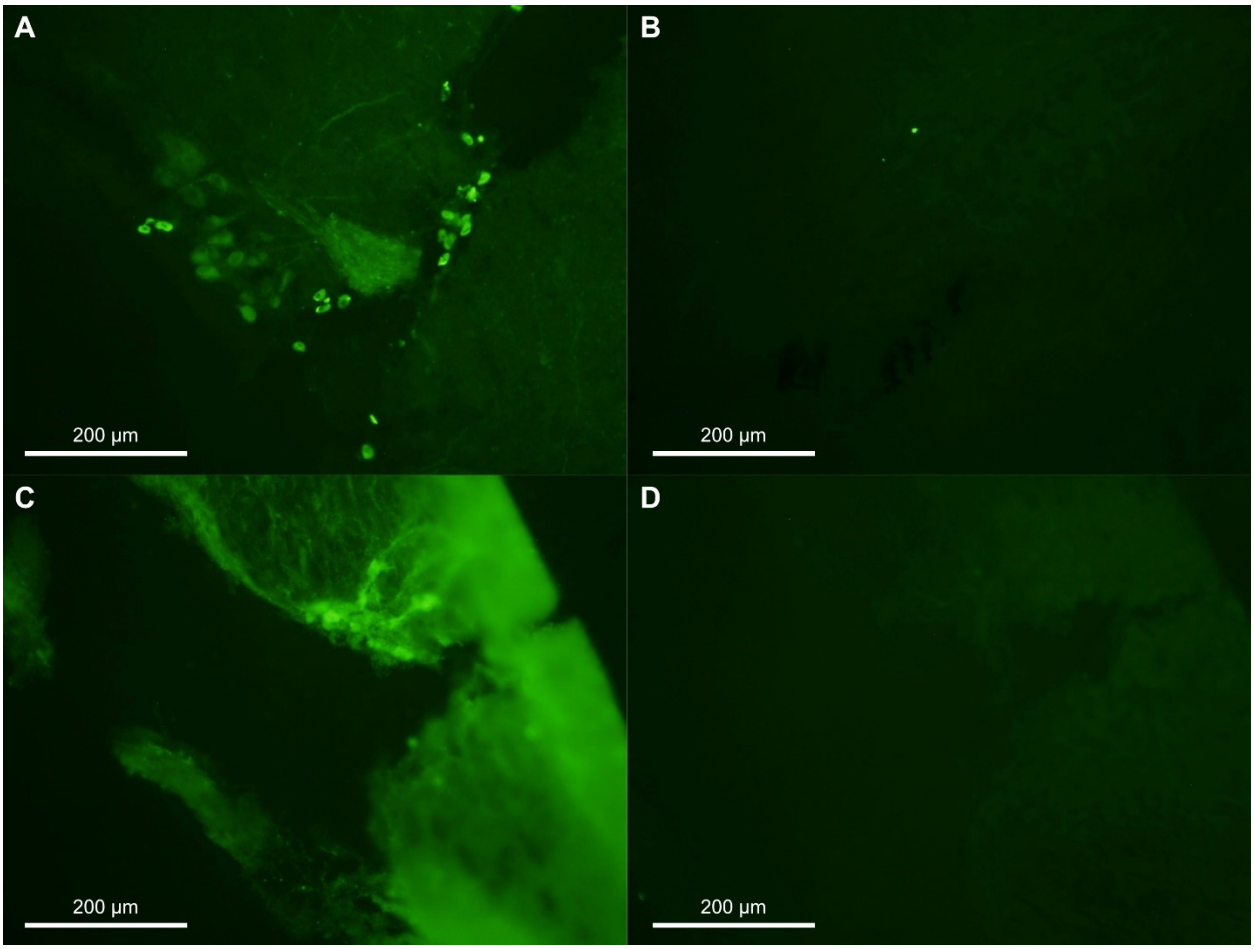

Fig. 4.4. Choline acetyltransferase immunohistochemistry in the brain and spinal cord of *Polypterus senegalus*. Adjacent sections (30 µm thick) from the area of the laterodorsal tegmental nucleus (A) with and (B) without primary choline acetyltransferase antibody show specific staining of neurons and neuronal tracts. Adjacent 30 um sections from the rostral spinal cord (C) with and (D) without primary choline acetyltransferase antibody showing specific staining of neurons and neuronal tracts. Choline acetyltransferase staining confirms that cholinergic neurons (commonly found in the mesencephalic locomotor region) are present in the brain and spinal cord of *P. senegalus*.

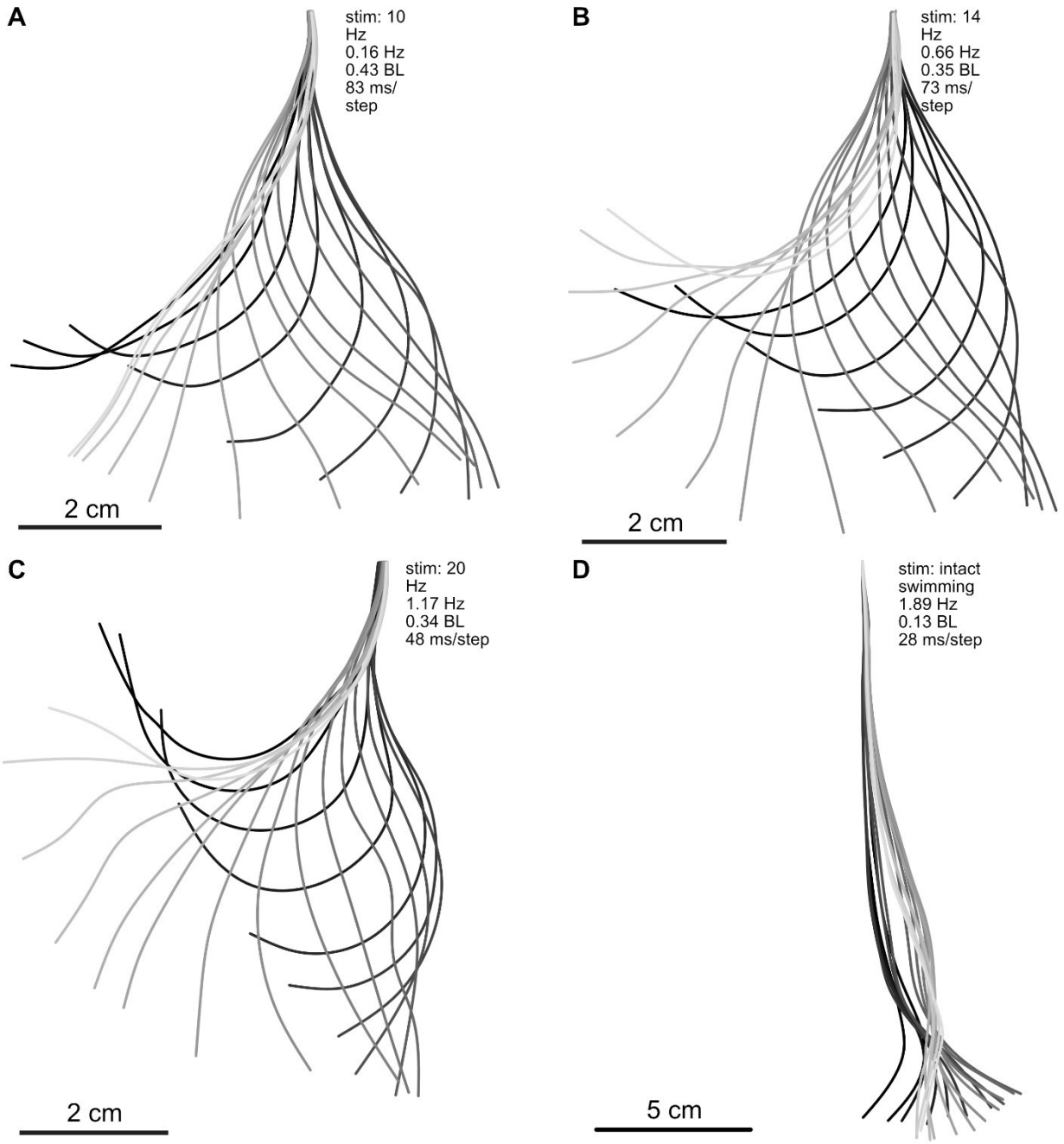

Fig. 4.5. Example midlines of swimming behaviour elicited by electrical stimulation of a putative mesencephalic locomotor region and normal swimming behaviour. (A-C) Midlines (from the anterior end of the pectoral fin to the tail) from a single *Polypterus senegalus* that demonstrated repeatable responses to stimulation of a putative mesencephalic locomotor region show an exaggerated amplitude and lower frequency than (D) normal swimming. (D) midlines from normal swimming were rotated and translated such that the head was in line throughout the swimming cycle to match the electrical stimulation preparation. Midlines are displayed from the anterior end of the pectoral fins to the tail to match midlines in (A-C). Twenty midlines through a tailbeat cycle are shown in each panel, progressing from black to light grey. Stim – stimulation frequency.

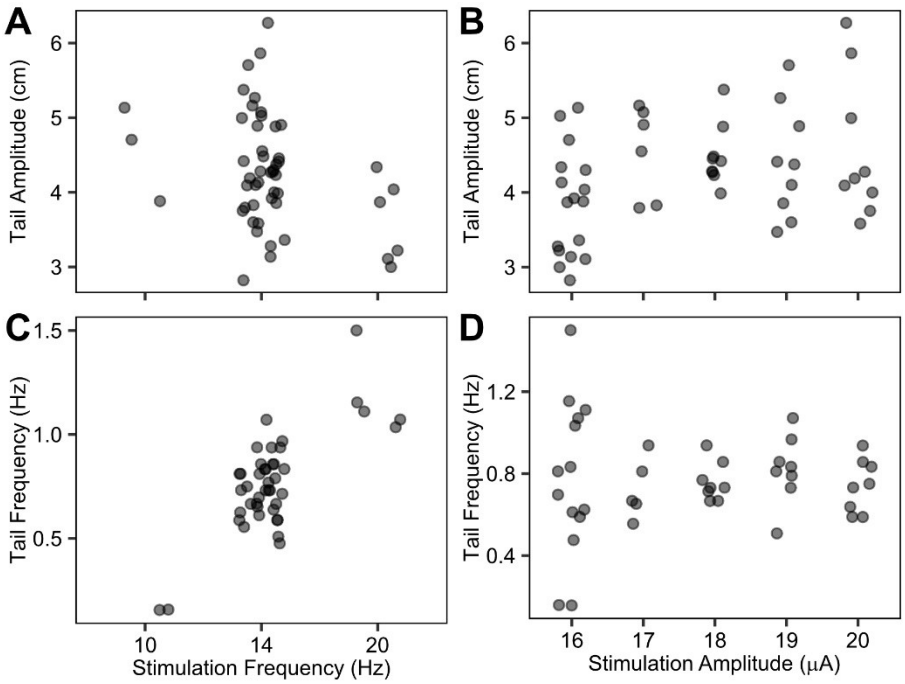

Fig. 4.6. Tail kinematics following stimulation of the putative mesencephalic locomotor region in a *Polypterus senegalus*. (A, B) Tail amplitude and (C, D) tail frequency are presented as a function of (A, C) stimulation frequency and (B, D) stimulation amplitude. Stimulation parameters are plotted in discrete steps with random jitter at each value to minimize overlap of points and facilitate visualization. Most trials in this preparation were performed at a stimulation frequency of 14 Hz because this elicited the strongest kinematic response.

4.8 References

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4.9 Supplementary Material

Matlab and R code for analysis and plotting can be found on GitHub.

CHAPTER 5: PLASTICITY OF MUSCLE ACTIVITY FOLLOWING TERRESTRIAL ACCLIMATION OF AN AMPHIBIOUS FISH

Chapter Notes

This chapter is in preparation to be submitted as a manuscript:

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Author contributions

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5.1 Abstract

Amphibious fish often rely on phenotypic plasticity to accommodate the dramatic physiological and biomechanical differences between aquatic and terrestrial locomotion. Behavioural plasticity may be especially important for accommodating novel environments since it can be immediate and is often reversible. Prior work in *Polypterus senegalus* has demonstrated *activational* behavioural and neuromuscular plasticity (over acute timescales) when fish are placed on land and *developmental* behavioural plasticity (over chronic timescales) when fish are raised on land. However, it is unclear how neuromuscular control is altered following terrestrial acclimation or whether dynamic loading of tissues (that results from locomotor movements) is essential to these *developmental* plastic changes. We hypothesized that previously demonstrated *developmental* plasticity results from changes in pectoral fin neuromuscular control and that dynamic loading was sufficient to generate such plastic changes. To test these hypotheses, we raised *P. senegalus* in aquatic, exercise (aquatic housing, 1 m daily walk), and terrestrial treatments for 10 weeks and recorded kinematics and muscle activity during walking following treatment. Walking behaviour differed between groups, primarily due to an increased nose elevation in terrestrial fish. Overall behaviour differences were smaller, but of a similar kind to those shown in the previous eight-month terrestrial acclimation. These behavioural differences were driven by shorter, more precise bursts of coracometapterygialis muscle activity that likely reduce friction between the pectoral fin rays and the ground and that plant the pectoral fin appropriately to lift the nose. Thus, refinement of walking neuromuscular control results from chronic terrestrial acclimation and to a limited extent dynamic loading, suggesting that these neuromuscular changes result primarily from static loading of the pectoral fins. Further, since subtle changes in muscle activity generate a more effective walk, we suggest that small changes in neuromuscular control may improve the terrestrial performance of amphibious fish.

5.2 Introduction

Plasticity is the ability to adjust biochemical, physiological, morphological, and/or behavioural phenotype in response to the environment (Wright and Turko, 2016) and may be especially critical for performance when an animal moves between dramatically different environments. Amphibious animals often utilize plastic changes to regulate ion and water balance, respiration and metabolism, acid-base balance, nitrogenous waste excretion, and physical forces in both aquatic and terrestrial environments (Wright and Turko, 2016). Because these environments pose dramatically different challenges to the animal, researchers have been interested in evolutionary transitions from water to land for decades (e.g. Edwards, 1989; Coates et al., 2008; Hsieh, 2010; Ahlberg, 2019; Standen et al., 2014; Dickson et al., 2021). That plasticity is part of the substrate upon which evolutionary processes can act is not a new idea (reviewed in West-Eberhard, 2003). Recent studies have highlighted both the importance of plasticity for acclimation to novel environments (e.g. Brunt et al., 2016; McFarlane et al., 2019; Standen et al., 2014; Jew et al., 2013), and the potential for plastic traits to become incorporated (fixed) in the genome and subject to adaptive selection (Pfennig et al., 2021). Adaptation to a novel environment requires changes at all levels of organization; however, behavioural plasticity, which can be immediate and reversable, may be an essential first step when gaining access to or experiencing new environments.

Behavioural plasticity can be categorized as *activational* or *developmental* (Snell-Rood, 2013). *Activational* behavioural plasticity is the result of selective activation of pre-existing neural networks to generate new behaviour. This is essentially "cryptic" behavioural variation. Much like cryptic genetic variation, *activational* behavioural plasticity is only expressed under a specific set of environmental conditions. Thus, *activational* behavioural plasticity is likely essential when an animal adapted for an aquatic environment first leaves the water to move on land. As an individual spends progressively more time on land, *developmental* behavioural plasticity, based on the formation of new

neural pathways or the refining of old pathways, may improve or fine tune locomotor behaviour so the animal is more effective at moving on land. Indeed, there are several examples of plastic improvements to terrestrial locomotion in amphibious fish following terrestrial acclimation (Brunt et al., 2016; McFarlane et al., 2019; Standen et al., 2014). If these changes in behaviour improve fitness in that new environment, they may help generate a positive feedback loop that could reinforce emersion behaviour (Crespi, 2004; Tigert et al., 2021 preprint). Thus, novel environments may express *activational* behavioural plasticity and provide a proving ground for refining (through developmental plasticity) and fixing new, effective behaviours into the genome (Pfennig et al., 2010; Pfennig, 2021).

5.2.1 Challenges of moving on land

In water, the body weight of the animal is often fully supported by buoyancy, and thus forward locomotion is only a matter of generating enough thrust to overcome drag and/or viscous forces, depending upon the speed and size of the animal (i.e. Reynold's number). In contrast, moving forward on land requires an animal to lift themselves off the substrate (against the weight of gravity) or exert additional force to overcome friction between the body and the ground. Since these two force environments place different demands on the body, an animal that has evolved entirely for effective aquatic locomotion is likely not well suited for a terrestrial environment. Additionally, walking on land, even for terrestrial-adapted animals, tends to be more energy expensive than swimming (Schmidt-Nielsen, 1972). This combination of moving in an environment to which they are not solely adapted and the general high cost of terrestrial transport means that fish muscles must work harder to generate locomotion in a terrestrial environment (Foster et al., 2018; Gillis, 2000; Horner and Jayne, 2014; Pearlman and Ashley-Ross, 2016).

The increased muscle effort that is necessary to facilitate forward locomotion in a terrestrial environment is exercise. In mammals, high-intensity, anaerobic exercise favours hypertrophy of (and

potentially muscle fiber transitions to), "white", fast contracting, high force, glycolytic fibers (McClelland, 2012). Aerobic exercise, on the other hand, favours hypertrophy of (and potentially muscle fiber transitions to) "red", slow contracting, low force, fatigue resistant fibers. In fish, the effect of swimming exercise varies between species, but generally accords well with the mammalian pattern. Swim training at routine speeds over long durations leads to an increase in red muscle cell number and size as well as an increase in the size of white muscle fibers, even at low speeds (Davison, 1997; McClelland, 2012). The effect of terrestrial exercise on fish behaviour and muscle phenotype seems to be more variable. Air-acclimated (but not exercised) mangrove rivulus (*Kryptolebias marmoratus*) had better jumping performance and larger diameter axial red muscle fibers (but the same number of fibers) than their aquatically raised counterparts (Brunt et al., 2016) and terrestrial exercise in mangrove rivulus further improves performance and increases axial red muscle fiber diameter (McFarlane et al., 2019). Conversely, terrestrially-acclimated *Polypterus senegalus* (free to move voluntarily) have a higher number of "fast" pectoral fin muscle fibers in all pectoral fin muscles following two months on land (with no clear trend for fiber size change) relative to their aquatically-reared counterparts (Du and Standen, 2017). These different plastic responses could be the result of many things including phylogeny, how well adapted each species is to a terrestrial environment, mode of locomotion, and size, but all responses to terrestrial acclimation or exercise improved terrestrial locomotion (Brunt et al., 2016; McFarlane et al., 2019; Standen et al., 2014).

Polypterus senegalus is a growing model to investigate the role of plasticity in the transition from aquatic to terrestrial life history for several reasons. First, the pectoral fin is made of four muscle groups that may permit behavioural and functional flexibility (Wilhelm et al., 2015) and is comparable to early tetrapod ancestors, the elpistostegid fishes (Clack, 2012). The abductor and adductor move the pectoral fin away from and towards the body, respectively. The zonopropterygialis and coracometapterygialis lift and lower the pectoral fin (respectively) and can

also contribute to rotation. Second, following several months of terrestrial acclimation, *P. senegalus* exhibit pectoral girdle bone plasticity that mirrors the evolutionary transition from fish to tetrapod (Standen et al., 2014) and increased ossification of the pectoral fin radials that may assist locomotor performance (Du and Standen, 2020). Finally, these fish are capable of tetrapod-like terrestrial locomotion that relies upon the pectoral fins bearing weight. This terrestrial locomotion becomes more effective following terrestrial acclimation, due to higher nose and pectoral fin elevation and smaller tail excursions, facilitated by pectoral fins being planted closer to the body midline, less pectoral fin slip during stance, and more precisely timed body and pectoral fin kinematic events (Standen et al., 2014). This terrestrial plasticity is all despite there being few actual observations of voluntary emersion in the wild or in lab (Du et al., 2016).

Polypterus senegalus therefore posses an innate ability to walk (*activational* behavioural plasticity) in a terrestrial environment which improves following long-term (months) terrestrial acclimation (*developmental* behavioural plasticity). Because behavioural plasticity is dependant upon neuromuscular organization and control (Mery and Burns, 2010) and because changes in physiology can improve behavioural performance (e.g. Brunt et al., 2016; McFarlane et al., 2019; Standen et al., 2014), understanding the neuromuscular mechanisms that underpin *developmental* behavioural plasticity is essential to understanding the extent of physiological change required to facilitate adaptive behavioural plasticity. Since previous work has demonstrated that terrestrial acclimation in *P. senegalus* generates behavioural plasticity and terrestrial exercise in the absence of chronic terrestrial acclimation in mangrove rivulus generates changes in muscle morphology and jumping performance, we ask two questions: (1) is muscle activation altered by terrestrial acclimation and (2) is terrestrial exercise without chronic terrestrial exposure sufficient to elicit these changes?

We test two hypotheses using *P. senegalus*. First, we hypothesize that more effective walking in terrestrially acclimated *P. senegalus* is the result of changes in pectoral fin muscle activity. This

predicts: (1) that abductor intensity increases in terrestrially-acclimated fish, since this muscle may be involved in lifting the nose during the stance phase, (2) coracometapterygialis intensity increases in terrestrially-acclimated fish to keep the fin planted close to the midline of the body and help lift the nose, (3) that zonopropterygialis intensity increases in terrestrially acclimated fish to lift the pectoral fin higher off the ground during swing and (4) that muscle activity timing is less variable in terrestrially acclimated fish to create more predictable kinematic timing. Second, we hypothesize that terrestrial exercise alone is sufficient to generate behavioural and muscle activity changes in *P. senegalus*. This predicts that the behaviour and muscle activity of terrestrially-exercised fish raised in water will be similar to that of terrestrially-acclimated fish.

5.3 Materials and Methods

5.3.1 Animals, acclimation, and surgical procedure

Polypterus senegalus (n=13) were obtained from the pet trade (Mirdo Importations Canada, Montreal, QC, Canada) and acclimated to lab conditions in individual flow through tanks (26-28 °C; 12/12 light/dark cycle) for at least two weeks prior to the beginning of treatment. Fish were housed individually and randomly assigned to control (n=4), exercise (n=5), and terrestrial (n=4) groups. Treatment conditions were like Du and Standen (2020). Briefly, control fish and exercise fish were kept in aquatic conditions as described above. Control fish were subjected to aerial net stress for 30 seconds each day to match the handling of the exercise group. Exercise fish were netted and exercised (1 m terrestrial walk along a ramp with a slight decline) daily to induce dynamic loading of the pectoral fins. Terrestrial fish were kept in terrestrial conditions (1 mm of water above a gravel substrate) like previous studies (Du and Standen, 2017; Du and Standen, 2020; Standen et al., 2014). The water level in the tanks of the terrestrial fish was raised for 30 minutes daily to allow feeding and cleaning. During this time terrestrial fish were exposed to aerial net stress as for the control fish.

Fish mass and total length were recorded at the beginning of treatment (11.3 ± 0.44 g; 120.2 ± 9.3 mm), just before recording baseline swimming and walking kinematics (without electromyography (EMG) wires implanted; 10.4 ± 0.4 g; 108.6 ± 11.8 mm), and after recording swimming and walking with electromyography wires implanted (10.8 ± 0.4 g; 119.0 ± 9.0 mm). To quantify the distance fish moved in the terrestrial condition, and better understand the relative importance of static and dynamic terrestrial loading for fish in this condition, we recorded six periods of at least 20 hours for each of the terrestrial fish and measured the distance they moved during that time. Fish were placed into treatments in cohorts of three (one fish in each treatment). Fish were fed daily with sinking fish pellets and beef heart. To keep growth as similar as possible between treatment groups, each cohort of fish was fed only if the terrestrial fish in that cohort ate. While there were no differences in mass between treatments (linear mixed effects model: $F_{2,10}$ = 1.35; p = 0.30), fish tended to weigh less as the experiment progressed (linear mixed effects model: $F_{2,24}$ = 29.68, p <0.0001).

5.3.2 Experimental protocol

Following 10 weeks in treatment, fish were anaesthetized in buffered 300 mg/L MS222 (tricaine methanesulfonate) until breathing slowed and they were unresponsive to touch. Fish were then implanted percutaneously with four fine-wire two-pronged hook electrodes (0.051 mm insulated bi-filament stainless steel wire; California Fine Wire Company, Grover Beach, CA, USA) in the abductor, adductor, coracometapterygialis, and zonopropterygialis of the right pectoral fin using sharp 30-gauge needles (1.5 inch barrel for the abductor and adductor and 1 inch barrel for the coracometapterygialis and zonopropterygialis) (Fig. 5.1). While both the coracometapterygialis and adductor have two heads (Wilhelm et al., 2015), we treat these here as a single muscle. As in Lutek and Standen (2021), we also implanted seven electrodes in red axial muscle (Fig. 5.1); five along the left side spaced equidistantly between the posterior edge of the pectoral fin lobe and anterior edge of

the anal fin (average positions (% BL): 22.5 ± 0.1, 36.4 ± 0.1, 48.3 ± 0.2, 62.7 ± 0.2, 73.5 ± 0.2) and two on the right side opposite positions three and five (average positions (% BL): 49.6 ± 0.2, 74.3 ± 0.2) using 27-gauge needles. Wires were secured to the dorsal finlets with suture to reduce strain. Fish recovered in fish housing water for at least 30 minutes following surgery.

Following recovery, we recorded high-speed kinematics and muscle activity for bouts of steady walking consisting of at least three kinematic cycles until each fish had completed a total of ten or more kinematic cycles. Kinematic and muscle activity timing was synchronized using an external trigger. Kinematics were recorded at 500 fps in dorsal and lateral views using two Photron FastCam Mini UX (Photron USA Inc., San Diego, CA, USA). Cameras were calibrated using easyWand to allow 3D position to be calculated. All trials were run at room temperature (23-24 °C). We simultaneously recorded muscle activity signals amplified 5000 times by a Grass P511 AC Amplifier (Grass Instrument Company, West Warwick, RI, USA), converted from analog to digital by a Powerlab 16/35 (AD Instruments, Colorado Springs, CO, USA), and recorded in Lab Chart 8 (AD Instruments). We recorded signals at 10 kHz with a 60 Hz notch filter to reduce ambient electrical noise. Following recording of normal swimming and walking, fish were euthanized with an overdose of buffered MS222 (417 mg/L). We then recorded mass and total length and confirmed electrode position under a dissection microscope.

5.3.3 Kinematics analysis

For all trials the nose, tail, right fin, and back of skull were digitized in 3D using DLTdv8 (Hedrick, 2008) in Matlab (version R2021a, The MathWorks, Nantick, MA, USA). Coordinates were then filtered with a low-pass filter (cutoff was five times movement oscillation frequency for each point) to remove digitizing error. All length measurements were standardized to body length or pectoral fin length (as in Standen et al., 2014) to account for any inter-individual size differences. We focus here on muscle activity changes that could generate the more effective walking behaviour

previously described in fish acclimated to terrestrial environments (Standen et al., 2014). We therefore calculated a set of 'key' kinematic variables from Standen et al. (2014) to determine whether 10 weeks of terrestrial exercise or terrestrial acclimation were sufficient to generate similar kinematic changes. We selected nine kinematic variables from Standen et al. (2014) for preliminary analysis: step duration (s), stance time (s), pectoral fin slip time (s), abduction and adduction distance (BL), pectoral fin elevation (BL), nose elevation (BL), pectoral fin slip distance (BL), tail swing distance (BL) and perpendicular distance between the tip of the pectoral fin rays and the body midline (BL; fin-midline distance). Using FIJI (Schindelin et al., 2012) we manually identified the beginning and end of stance and the beginning and end of slip. From these values we calculated step duration, stance time, and pectoral fin slip time. Abduction and adduction distance were calculated as the total distance travelled by the pectoral fin tip during swing and stance, respectively. Pectoral fin and nose elevation were calculated as the difference between maximum elevation and the subsequent minimum elevation for each point. Pectoral fin slip distance was calculated as the total distance travelled by the tip of the pectoral fin rays during slip. Tail swing distance was the total distance travelled by the tip of the caudal fin from a maximum amplitude to the left and a subsequent maximum amplitude to the right. Minimum fin-midline distance was calculated as the minimum perpendicular distance in the x-y plane between the tip of the pectoral fin rays and a line from the tip of the nose to the first dorsal spine during stance. To permit linear discriminant analysis (LDA), we sorted each of these values into the step during which the event occurred. If more than one event occurred within a step, we used the average value for that variable. We report the timing of maximum nose elevation, maximum tail swing to the left, minimum fin-midline distance, and the start of pectoral fin slip in polar coordinates relative to the nose swing cycle. These variables were selected to represent when the four most important magnitude variables (based on the results of the LDA; see 'Statistical analysis' below) occurred within a walking step.

5.3.4 Electromyography analysis

Muscle activity signals were filtered using a 40 – 4000 Hz bandpass signal to remove movement artifacts and electrical noise (Foster et al., 2018; Lutek and Standen, 2021). We visually identified muscle onset and offset timings using custom Matlab code, using twice the standard deviation of a baseline signal as a guide for when muscles were active. Using those onset and offset timings, we calculated burst duration (s), muscle activity duty factor (% kinematic cycle), and muscle activity rectified integrated area (% theoretical maximum) as in Chapter 3. To identify changes in muscle activity timing, we report muscle activity onset and offset in polar coordinates relative to the nose swing cycle.

5.3.5 Statistical analysis

All linear statistics and graphing were performed in RStudio (RStudio Team, 2021) using the tidyverse package (Wickham et al., 2019). Linear mixed effects (LME) models were calculated using nlme (Pinheiro et al., 2022). LME results are reported in ANOVA-type tables for simplicity. When necessary, pairwise comparisons are carried out based on estimated marginal means (Lenth, 2022) for each model and Bonferroni-corrected within each variable of interest to account for type I error.

To determine overall changes in walking kinematics between treatments we used an LDA, which determines the axes that create the most separation between treatment groups. Prior to performing the LDA, we checked for co-linearity of kinematic variables and removed those that were more than 80% correlated with each other. This left us with seven variables that represent the overall changes in walking between treatments: stroke duration, adduction distance, pectoral fin elevation, slip distance, nose elevation, tail swing distance, and fin- midline distance. These variables were fed into a LDA to determine kinematic differences between groups. We report the LDA weightings as a qualitative representation of the importance for variables in describing differences between treatments. To determine specific changes in walking behaviour we generated LME models

for the three variables that loaded most heavily on each of the two LDA axes with treatment as a fixed effect and a random intercept determined by trial nested inside individual. Differences in muscle activity between treatments and muscles were tested using LME models with fixed effects of treatment and muscle and a random intercept term of trial nested inside individual.

We tested differences in mean kinematic and muscle activity timing and timing variability using standard polar statistics (after Standen et al., 2014, Chapter 3) based on Zar (1999) and Baschelet (1981). For each variable, we tested for a von Mises distribution across all strokes, determined directionality using Rayleigh's test ($p < 0.05$), and tested for differences in angular mean (Watson-Williams test) and angular dispersion (Kruskal-Wallis test) between treatments if these assumptions were met. Polar statistics were carried out in Matlab using custom code and the Circular Statistics Toolbox (Berens, 2009).

5.4 Results

5.4.1 Acclimation affects walking kinematics

Since prior work has already detailed the kinematic changes in walking after acclimation to a terrestrial environment (Standen et al., 2014), we focus here on providing evidence for similar changes after a shorter acclimation (10 weeks) as well as for fish held in water but exposed to terrestrial exercise. On average, terrestrial fish voluntarily moved 3.27 m each day during the last week of acclimation, significantly more than the 1 m that exercise fish were required to walk ($t_4 = 3.54$; $p = 0.0014$). LDA of a set of seven uncorrelated kinematic variables identified as important in that prior investigation (Standen et al., 2014) suggest differences existed in walking behaviour between control, exercised, and terrestrial fish at the end of the acclimation period (Fig. 5.2A, Table S5.1). Univariate analyses of the three variables with the highest weightings on each of the two linear discriminant axes demonstrated a nominally significant increase (pairwise comparisons are not significant after Bonferroni correction) in nose elevation in terrestrial fish above that of control and

exercised fish (Fig. 5.2B, Tables 5.1, 5.2, S5.2), but no changes in tail swing distance, fin-midline distance, or fin swing distance (Fig. 5.2C,D,E, Tables 5.1, 5.2, S5.2). Collectively, this suggests there was indeed an effect of both exercise and terrestrial acclimations, although the behavioural differences were smaller than after 8 months, and that changes in terrestrial fish were in the same direction as those that improved the effectiveness of walking after 8 months of terrestrial acclimation.

There were no statistically significant changes in mean angular timing of maximum nose elevation or the start of tail swing to the right (Fig. 5.3A,B, Tables 5.3, 5.4, S5.3). The angular dispersion of the start of tail swing to the right was significantly lower in terrestrial fish than control fish (Fig. 5.3B, Tables 5.3, 5.4, S5.3). Minimum fin-midline distance occurred significantly earlier in exercised fish than in control or terrestrial fish (Fig 5.3C, Tables 5.3, 5.4, S5.3). Pectoral fin slip started significantly later in the kinematic cycle in terrestrial fish than exercised fish (Fig. 5.3D, Tables 5.3, 5.4, S5.3).

5.4.2 Acclimation alters muscle activity duration and timing

Our results found no significant changes in body muscle activity between treatments (Tables S5.4, S5.5). While there were also no significant changes in pectoral fin RIA (Fig. 5.4, Tables 5.1, 5.2, S5.2), there was a statistically significant decrease in coracometapterygialis muscle activity duration and duty factor in terrestrial fish relative to control fish (Fig. 5.4B, Tables 5.1, 5.2, S5.2). Abductor muscle onset and offset occurred at predictable points in the walking cycle, but muscle onset did not meet the required assumptions to test for differences in timing or dispersion (Fig. 5.5A, Tables 5.3, 5.4, S5.3). Abductor muscle offset occurred significantly earlier in the walking cycle for exercised fish than control or terrestrial fish and was less variable in terrestrial fish than exercised fish (Fig. 5.5A, Tables 5.3, 5.4, S5.3). Adductor muscle onset was significantly later in the kinematic cycle for both exercised and terrestrial fish than control fish (Fig 5.5B, Tables 5.3, 5.4, S5.3). Adductor muscle

offset occurred significantly later in terrestrial fish than in exercised fish (Fig. 5.5B, Tables 5.3, 5.4, S5.3). Coracometapterygialis muscle onset shifts progressively earlier in the kinematic cycle between control, exercised, and terrestrial fish, and is more variable in exercised fish than control or terrestrial fish (Fig. 5.5C, Tables 5.3, 5.4, S5.3). There were no significant differences in coracometapterygialis offset timing or variability (Fig. 5.5C, Tables 5.3, 5.4, S5.3). Zonopropterygialis muscle onset showed no significant differences in timing or variability but offset happened significantly earlier and was more variable in terrestrial fish than control and exercised fish (Fig 5.5D, Tables 5.3, 5.4, S5.3).

5.5 Discussion

Here, we have investigated the capacity for terrestrial exposure and exercise to generate muscle activity plasticity that results in a more effective walking gait (like that demonstrated in Standen et al., 2014). While the changes in this study are smaller in magnitude, they are in line with previous plasticity seen in longer term terrestrially acclimated animals (Standen et al., 2014). Behavioural changes appear to result from with statistically significant changes in the duration and timing of the coracometapterygialis muscle activity in terrestrial acclimated fish indicating a more precise burst timing. These changes likely ensure that the pectoral fin is placed on the ground only when necessary, reducing the magnitude of frictional forces between the fin rays and the substrate that occurs during swing prior to the beginning of stance.

5.5.1 Activity of P. senegalus on gravel

Fish reared in a terrestrial environment with a gravel substrate moved on average three times more than the imposed exercise regime. This was an unexpectedly high level of movement for *P. senegalus* since they are predominantly aquatic and previous reports suggest that fish move very little when acclimating to a terrestrial environment (Du and Standen, 2020). Like the previous investigation our fish spent most of their time on land stationary (generally under their house);

however, when the fish did venture out into the rest of their tank, they moved across an impressive distance. Because of the size of the gravel, fish in the terrestrial treatment likely used a slithering-type gait that does not engage fins and therefore the fins were not loaded during this movement (gait I; Standen et al., 2016). This means that while fish in our terrestrial treatment were active, the forces directly experienced by the pectoral fins of the terrestrial acclimated fish would have primarily been static and so the terrestrial and exercise treatments likely still represent static and dynamic loading of the pectoral fins respectively.

5.5.2 The pace of behavioural plasticity

Although the results of our LDA suggest that there are indeed behavioural differences between each of the acclimation treatments, the only notable kinematic difference between groups within a single variable was a marginally significant increase in nose elevation of the terrestrially acclimated fish relative to the control and exercise fish (Table 5.3). On the other hand, pectoral fin muscle activity showed statistically significant differences between treatment groups. Since in this experiment the duration of acclimation was longer than required to find statistically significant differences in pectoral fin bone and muscle morphology (5 weeks - Du and Standen, 2017; 2020) but shorter than for the acclimation that generated clavicle-cleithrum plasticity and larger behavioural plasticity (in similar terrestrial conditions, Standen et al., 2014), this begs the question of whether morphology follows or enables behavioural plasticity. It appears from the data presented here, that neuromuscular plasticity, or the 'improvement' of neural control of a novel behaviour, occurs before (or at least to a greater degree in the same time frame) behavioural plasticity. In essence, a cascade of events drive the plastic response. Novel behaviour on the short-term (*activational* behavioural plasticity) influences mechanical loading on an animal. Changes in mechanics elicit a longer-term plastic response in behaviour (*developmental* behavioural plasticity), neuromuscular control, and muscle and bone remodelling.

Younger individuals are often more plastic than sexually mature individuals. While we are unable to age the fish in this or previous studies (and both studies used juvenile *P. senegalus* with no external indicators of adulthood), size generally correlates well with age in this species. Because of the nature of electromyography experiments, we selected animals that were as large as possible to avoid hampering walking performance due to the electrode tether. This means that the fish in the present study were larger than those in all previous investigations of terrestrial plasticity in *P. senegalus*, and therefore were likely older. The lesser extent of behavioural plasticity our fish displayed in response to a terrestrial environment compared with the previous literature could therefore be due to a combination of the time course of behavioural plasticity and the capacity for plastic changes of each developmental stage.

Based on the present evidence and those of previous studies, it seems likely that neuromuscular changes occur first. One main driver of fiber type changes is altered neuromuscular intensity and/or timing, as occurs during exercise (McClelland, 2012). Since our fish were raised in an aquatic environment prior to being placed in experimental treatment, fish in both the exercise and terrestrial acclimation groups were likely naïve walkers prior to being placed in their treatments. Previous studies suggest that captive and wild-caught *P. senegalus* of various sizes demonstrate *activational* behavioural plasticity when placed on land (Du et al., 2016; Foster et al., 2018; Standen et al., 2014; Standen et al., 2016). The observed changes in neuromuscular control between fish in aquatic and terrestrial environments generate marked changes in kinematics between swimming and walking (Du et al., 2016; Foster et al., 2018; Standen et al., 2014). These neuromuscular changes appear to be modifications of swimming axial neuromuscular control rather than a novel motor control pattern, at least for the body motion (for details on fin control see Chapter 3). Following terrestrial acclimation, changes in muscle fiber type and ossification of pectoral fin bones may be in direct response to the static loading of gravity. The rates at which motor control and morphology

change after exposure to a novel environmental condition may elucidate what drives each change. It appears from the longer-term terrestrial acclimation applied in this study that motor control changes occur faster and to a greater degree then kinematic changes. We did not quantify tissue morphology changes here, but the fact that kinematic changes lag behind motor control change here suggests that tissue morphological change is required to implement visible changes in kinematics (since previous shorter terrestrial acclimation generates morphological changes (Du and Standen, 2017; 2020) and thus may also lag behind motor control changes.

In the present study, we see subtle alterations in neuromuscular control that fine tune the timing of pectoral fin muscle contractions in order generate a more effective walking behaviour. We suggest that after eight months on land, the marginal neuromuscular differences seen here (e.g. RIA changes, other duty factor changes) may have been sufficiently reinforced to generate the larger magnitude behavioural differences observed in previous study (Standen et al., 2014). While any concrete link to performance or fitness has yet to be established for this scenario, all of the plastic changes described likely support the ability of *P. senegalus* to move effectively on land. In mangrove rivulus, a trade-off between aquatic and terrestrial performance appears to drive increased emmersion behaviour (Tigert et al., 2021 preprint). Although this is not the case in *P. senegalus* (Standen et al., 2014), improved locomotor performance could be a part of a suite of physiological, morphological, and behavioural changes that promote terrestrial excursions.

5.5.3 Static vs dynamic loading

Our results highlight the impact that static loading can have on behavioural and neuromuscular plasticity. While fish were not stationary in our terrestrial acclimation treatment, direct loading of the pectoral fin would have been rare. In contrast, our terrestrial exercise treatment exposed fish to one meter of walking daily (nearly seven body lengths), but little static loading, since fish were in an aquatic environment save for the 30 seconds required to do this walk. As in previous

work investigating the influence of terrestrial exercise on pectoral fin bone plasticity (Du and Standen, 2020), we observed a smaller plastic response in the exercised fish compared to the terrestrial fish. Although the overall behaviour of exercised fish had a noticeable difference from our control fish (based on the LDA results), the kinematic magnitude and timing of individual variables was not significantly different between these groups. However, we did see a step-wise reduction in the duty factor of the coracometapterygialis and hints of a stepwise increase in abductor RIA between control, exercised and terrestrial fish. This suggests that while terrestrial exercise may have some marginal effects on neuromuscular control, exposure to a terrestrial environment (at least on this timespan) is essential to generating a robust neuromuscular response and a more effective walking behaviour. With the new understanding of how active *P. senegalus* are in a gravel terrestrial environment, it would be interesting to repeat this experiment to see whether a more closely matched level of terrestrial walking in the exercise group could elicit a more robust behavioural and neuromuscular response or whether there are simply limits to the influence of dynamic loading.

These results could be interesting in an evolutionary context because they suggest that amphibious fish need not be very active initially to benefit from improved terrestrial performance, assuming they can survive a terrestrial environment. Terrestrial fish whose pectoral fins were primarily exposed to static gravitational loads still had changes in pectoral fin neuromuscular control and walking kinematics that generated a more effective gait. While previous work in the amphibious mangrove rivulus suggests that terrestrial exercise does indeed improve terrestrial performance (as measured by distance travelled), static acclimation to a terrestrial environment also improved the locomotor performance above aquatically reared fish (Brunt et al., 2016; McFarlane et al., 2019). Notably, these metrics of performance have more to do with endurance than the specific kinematics of the behaviour. While the changes associated with a more effective walking gait in *P. senegalus* theoretically reduce the thrust requirements for generating locomotion (as they tend to reduce

friction between the ventral surface of the body and the substrate below), they may require greater muscle force production (RIA here) and a link between these kinematic differences and any improvement in endurance has not been tested. Future studies could look at whether the time to fatigue differs between exercised and terrestrial acclimated fish, since both treatment groups appeared to have improved endurance during walking filming in the present study (K.L. pers. obs.). It could be that the exercise and terrestrial fish are accomplishing a similar functional result (i.e. greater terrestrial endurance) through different neuromuscular and behavioural changes. Additionally, a suite of physiological changes accompany the behavioural changes seen in mangrove rivulus. Any of these physiological changes could also be occurring in *P. senegalus*. Improvements in physiological performance (oxygen delivery, muscle fiber type, waste clearing) could preclude the need for behavioural plasticity if they facilitate similar endurance without a change in muscle fiber type or behavioural changes that lift the nose higher off the ground, both of which are likely energy-expensive prospects.

5.5.4 Implications for the initial steps of fish on land

Taken together, our results demonstrate that both static and dynamic loading in a terrestrial environment can induce plastic changes in neuromuscular control and behaviour. We suggest that both *activational* plasticity in response to an acute change from aquatic to terrestrial environment and *developmental* plasticity, resulting from longer term static or dynamic loading, are likely critical pieces to the biomechanical adaptations that permit terrestrial forays. The present work adds to a body of evidence for a particular series of events that facilitate and improve the musculoskeletal and behavioural phenotype of *P. senegalus*. While this is only one particular scenario that suggests the potential importance of *developmental* plasticity for accommodating locomotion in a novel environment, more precise pectoral fin muscle activity likely improves the terrestrial performance of

P. senegalus. If any of these changes are incorporated into the genome then they may help lead evolutionary change.

5.6 Figures & Tables

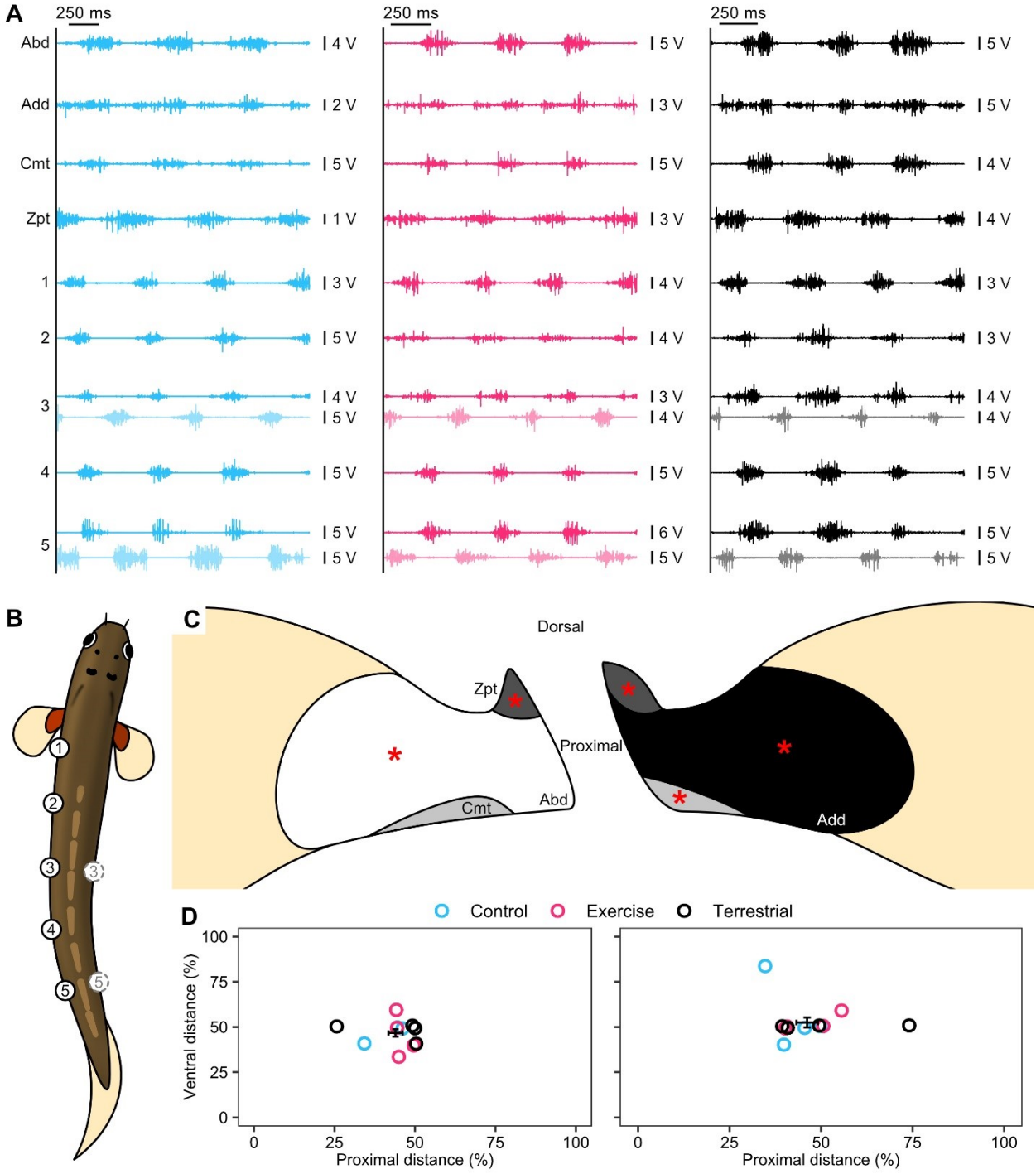

Figure 5.1. Body and fin electrode placement for walking trials of control, exercised and terrestrial *P. senegalus*. (A) Example EMG traces for control (blue), exercised (pink) and terrestrial (black) fish. Left side body electrodes are shown in full colour; right side electrodes are placed directly under the matching left side trace in half colour. (B) Body electrodes were placed in axial red muscle at the indicated positions. Left side electrodes are in black, right side electrode

positions are in grey. (C) Pectoral fin electrodes were placed in all four muscle groups. Red asterisks indicate the approximate position of each electrode in the muscles of interest. (D) Position of hook electrode tips in the abductor (left panel) and adductor (right panel). The cross of error bars indicate the grand mean and standard error in each direction. Points are the electrode position in each fish. Proximal distance was measured from the base of the fin lobe to the tip of the electrode. Ventral distance was measured from the ventral edge of the fin lobe to the tip of the electrode. Abd – abductor, Add – adductor, Cmt – coracometapterygialis, Zpt – zonopropterygialis.

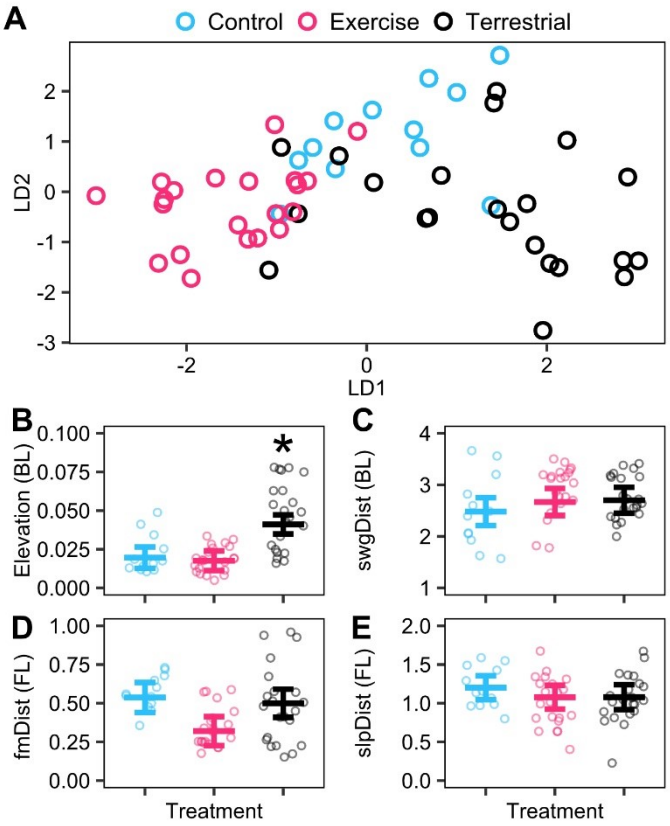

Figure 5.2. Kinematic changes following 10 weeks of terrestrial exercise or acclimation during walking in *P. senegalus*. (A) Linear discriminant analysis suggests that the kinematics of walking differs between control, exercised, and terrestrial *P. senegalus*. (B-E) Univariate tests for statistical differences in the three most heavily weighted variables on each linear discriminant axis. (B-D) load most heavily on linear discriminant axis one; (C-E) load most heavily on linear discriminant axis two. Loading scores can be found in Table S1. Marginally significant differences in estimated marginal mean from the control are indicated by an asterisk. Points in all panels are individual observations. Estimated marginal means ± their standard error are indicated in (B-D) with a horizontal line and error bars. BL, body length; FL, fin length; fmDist, fin-midline distance during stance; LD, linear discriminant score; slpDist, slip distance during stance; swgDist, tail swing distance.

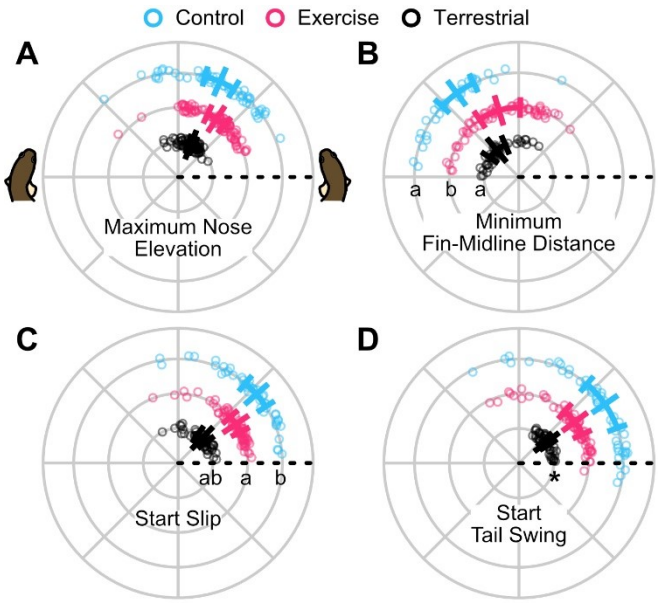

Figure 5.3. Kinematic timing changes during walking in exercised and terrestrial *P. senegalus*. Angular mean differences are indicated by letter reports only where significant differences exist. Asterisks denote a difference in angular distance from control fish. The start of the kinematic cycle (black dotted line) was defined as the nose starting to swing right; the middle of the kinematic cycle was defined as the nose starting to swing left (pictures in A). Points are individual observations. The angular mean ± angular variance is indicated by the vertical line and associated error bars.

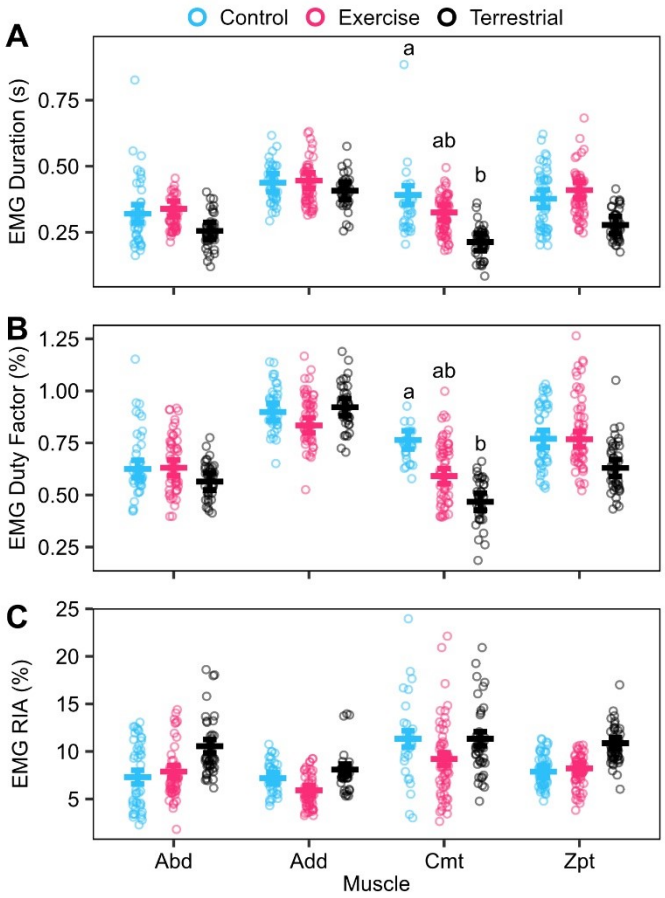

Figure 5.4. Muscle activity changes during walking in exercised and terrestrial *P. senegalus*. (B) EMG duty factor is shown as a percentage of the kinematic cycle duration. (C) EMG RIA is shown as a percentage of a theoretical maximum RIA (see text for details). Points are individual observations. Horizontal bar and associated error bars indicate the estimated marginal mean and its standard error. Significant differences in mean across treatments within a muscle are indicated by letter report when present. Abd – abductor, Add – adductor, Cmt – coracometapterygialis, EMG – electromyography, RIA – rectified integrated area, Zpt – zonopropterygialis.

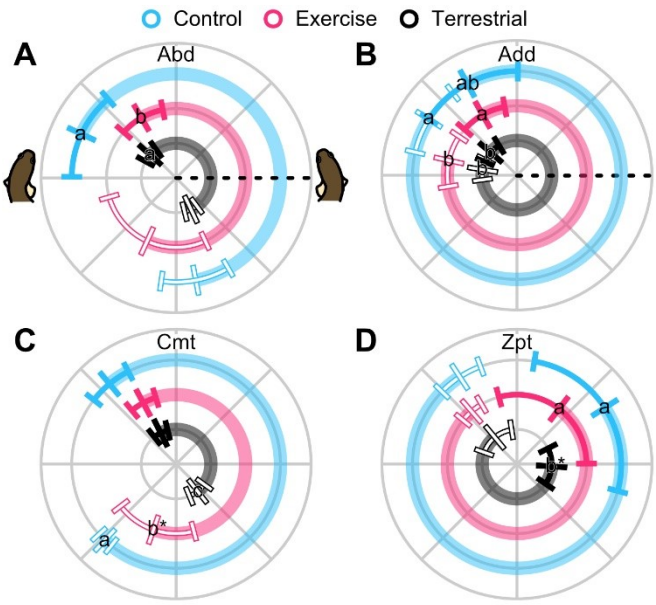

Figure 5.5. Muscle activity timing changes between exercised and terrestrial *P. senegalus* during walking. Onset (open points and bars) and offset (closed circles and bars) for each of the four pectoral muscle groups recorded during walking. Points are individual observations. Vertical bar and error bars indicate the angular mean and associated angular variance. When present, differences in onset and offset angular mean between treatments within a muscle are indicated by letter reports on top of each mean. When present, differences in angular distance from control fish are indicated by an asterisk beside the appropriate mean. Abd – abductor, Add – adductor, Cmt – coracometapterygialis, Zpt – zonopropterygialis.

Table 5.1. Kinematic and muscle activity changes during walking in exercised and terrestrial fish.

	Treatment			Muscle			Treatment×Muscle				
Variable	F	df	p	F	df	p	F	df	p	R^2_m	R^2_c
Nose Elevation (BL)	4.26	2,9	**0.049**	-	-	-	-	-	-	0.33	0.66
Fin-midline Distance (FL)	1.52	2,9	0.27	-	-	-	-	-	-	0.17	0.81
Slip Distance (FL)	0.21	2,9	0.82	-	-	-	-	-	-	0.03	0.91
Tail Swing Distance (BL)	0.20	2,9	0.82	-	-	-	-	-	-	0.02	0.83
Pectoral Fin EMG Duration (s)	1.92	2,10	0.20	15.38	3,544	**<0.001**	11.49	6,544	**<0.001**	0.31	0.60
Pectoral Fin EMG Duty Factor (%)	0.85	2,10	0.46	39.22	3,492	**<0.001**	16.68	6,492	**<0.001**	0.49	0.65
Pectoral Fin RIA (%)	6.10	2,10	**0.02**	12.38	3,544	**<0.001**	6.38	6,544	**<0.001**	0.39	0.68

ANOVA-type summary statistics from linear mixed effects models for linear kinematics and pectoral fin muscle activity during walking. R^2_m, marginal R-squared; R^2_c, conditional R-squared.

Table 5.2. *Post-hoc* pairwise comparisons of kinematic and muscle activity differences during walking in exercised and terrestrial fish.

		Ctrl-Exer		Ctrl-Terr		Exer-Terr	
Variable	Muscle	p	p_b	p	p_b	p	p_b
Nose Elevation (BL)	-	0.84	>0.99	**0.045**	0.14	**0.027**	0.080
Fin-midline Distance (FL)	-	0.14	0.43	0.79	>0.99	0.20	0.61
Slip Distance (FL)	-	0.59	>0.99	0.60	>0.99	>0.99	>0.99
Tail Swing Distance (BL)	-	0.63	>0.99	0.57	>0.99	0.93	>0.99
Pectoral Fin EMG Duration (s)	Abd	0.69	>0.99	0.20	>0.99	0.086	>0.99
	Add	0.86	>0.99	0.54	>0.99	0.40	>0.99
	Cmt	0.18	>0.99	**0.004**	**0.049**	**0.028**	0.34
	Zpt	0.48	>0.99	0.060	0.72	**0.013**	0.16
Pectoral Fin EMG Duty Factor (%)	Abd	0.92	>0.99	0.32	>0.99	0.25	>0.99
	Add	0.27	>0.99	0.70	>0.99	0.14	>0.99
	Cmt	**0.012**	0.14	**<0.001**	**0.006**	**0.048**	0.57
	Zpt	0.97	>0.99	**0.035**	0.42	**0.030**	0.36
Pectoral Fin RIA (%)	Abd	0.56	>0.99	**0.009**	0.11	**0.018**	0.22
	Add	0.13	>0.99	0.31	>0.99	**0.020**	0.24
	Cmt	0.076	0.91	0.99	>0.99	0.056	0.67
	Zpt	0.67	>0.99	**0.005**	0.06	**0.008**	0.09

P-value differences for pairwise comparisons across treatments following linear mixed effects models. Significant p-values are in bold. Abd – abductor; Add – adductor; BL – body length; Cmt – coracometapterygialis; Ctrl – control; Exer – exercise; FL – fin length; Terr – terrestrial; p – uncorrected p-value; p_b – bonferroni corrected p-value; Zpt – zonopropterygialis.

Table 5.3. Differences in kinematic and muscle activity timing during walking between treatments.

		Watson-Williams			Kruskal-Wallis		
Variable	Muscle	F	df	p	χ^2	df	p
Max Nose Elevation (°)†	-	-	-	-	1.2	2	0.54
Min Fin-midline Distance (°)	-	7.9	2,133	**<0.001**	3.6	2	0.17
Start Slip (°)	-	4.1	2,161	**0.02**	1.2	2	0.54
Start Tail Swing (°)	-	0.2	2,164	0.86	8.0	2	**0.02**
Pectoral Fin EMG Onset (°)	Abd†	-	-	-	-	-	-
	Add	5.3	2,137	**0.006**	1.5	2	0.469
	Cmt	46.0	2,129	**<0.001**	28.3	2	**<0.001**
	Zpt	0.7	2,145	0.477	5.2	2	0.075
Pectoral Fin EMG Offset (°)	Abd	12.1	2,142	**<0.001**	8.8	2	**0.013**
	Add	4.1	2,134	**0.018**	0.9	2	0.640
	Cmt	2.2	2,132	0.112	3.5	2	0.175
	Zpt	12.1	2,145	**<0.001**	13.9	2	**<0.001**

† does not meet the assumptions of the Watson-Williams test. The Watson-Williams test looks for differences in angular mean between treatments, the Kruskal-Wallis test looks for differences in angular distance between treatments.

Table 5.4. Pairwise comparisons between treatments for polar kinematics and muscle activity during walking.

		Watson Williams						Kruskal-Wallis					
		Ctrl-Exer		Ctrl-Terr		Exer-Terr		Ctrl-Exer		Ctrl-Terr		Exer-Terr	
Variable	Muscle	p	p_b	p	p_b	p	p_b	p	p_b	p	p_b	p	p_b
Max Nose Elevation (°)†	-	-	-	-	-	-	-	-	-	-	-	-	-
Min Fin-midline Distance (°)	-	**0.003**	**0.009**	0.661	>0.99	**0.001**	**0.003**	-	-	-	-	-	-
Start Slip (°)	-	0.080	0.240	0.329	0.956	**0.005**	**0.016**	-	-	-	-	-	-
Start Tail Swing (°)	-	-	-	-	-	-	-	0.139	0.418	**0.015**	**0.044**	0.522	>0.99
Pectoral Fin EMG Onset (°)	Abd†	-	-	-	-	-	-	-	-	-	-	-	-
	Add	**0.008**	**0.025**	**0.003**	**0.009**	0.675	>0.99	-	-	-	-	-	-
	Cmt	**0.011**	**0.031**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.058	0.173	**0.005**	**0.014**
	Zpt	-	-	-	-	-	-	-	-	-	-	-	-
Pectoral Fin EMG Offset (°)	Abd	**<0.001**	**<0.001**	**0.024**	0.073	**0.005**	**0.014**	0.299	0.897	0.372	>0.99	**0.009**	**0.026**
	Add	0.893	>0.99	**0.021**	0.062	**0.007**	**0.022**	-	-	-	-	-	-
	Cmt	-	-	-	-	-	-	-	-	-	-	-	-
	Zpt	0.121	0.362	**0.002**	**0.005**	**<0.001**	**<0.001**	0.941	>0.99	**0.002**	**0.006**	**0.005**	**0.016**

† indicates when a given variable did not meet the assumptions for either test and so could not be assessed statistically. Dashes indicate where pairwise comparisons could /need not be performed. Significant p-values are in bold.

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5.8 Supplementary Materials

Table S5.1. Linear discriminant analysis of walking in control, exercise, and terrestrial acclimated *P. senegalus*.

Variable	Linear Discriminant Coefficient	
	LD1 (0.82)	LD2 (0.18)
Nose Elevation	**1.21**	-0.33
Tail Swing Distance	**-0.79**	**-0.70**
Fin-Midline Distance	**0.71**	**0.57**
Adduction Arc Length	0.48	-0.38
Fin Elevation	0.26	-0.005
Stroke Duration	-0.04	0.02
Slip Distance	0.015	**0.62**

Linear discriminant coefficients for each of the seven variables included in the linear discriminant analysis. Variables are ordered from highest absolute weighting to lowest absolute weighting on linear discriminant axis one. The proportion of trace accounted for by each linear discriminant axis is in brackets next to each heading. The top three weightings for each axis are in bold.

Table S5.2. Kinematic and pectoral fin muscle activity values during walking after exercise and terrestrial acclimation.

Variable	Muscle	Ctrl	Exer	Terr
Nose Elevation (BL)	-	0.020±0.007	0.018±0.006	0.041±0.006
Fin-midline Distance (FL)	-	0.54±0.10	0.32±0.09	0.50±0.09
Slip Distance (FL)	-	1.20±0.15	1.08±0.15	1.08±0.16
Tail Swing Distance (BL)	-	2.48±0.27	2.67±0.26	2.70±0.25
Pectoral Fin EMG Duration (s)	Abd	0.32±0.03	0.34±0.03	0.26±0.03
	Add	0.44±0.03	0.45±0.03	0.41±0.03
	Cmt	0.39±0.04	0.33±0.03	0.21±0.03
	Zpt	0.38±0.03	0.41±0.03	0.28±0.03
Pectoral Fin EMG Duty Factor (%)	Abd	0.63±0.04	0.63±0.04	0.57±0.04
	Add	0.90±0.04	0.83±0.04	0.92±0.04
	Cmt	0.76±0.04	0.59±0.04	0.47±0.04
	Zpt	0.77±0.04	0.77±0.04	0.63±0.04
Pectoral Fin RIA (%)	Abd	7.30±0.72	7.88±0.64	10.55±0.70
	Add	7.20±0.59	5.91±0.52	8.09±0.59
	Cmt	11.33±0.85	9.21±0.65	11.34±0.74
	Zpt	7.88±0.59	8.22±0.54	10.87±0.59

Estimated marginal mean ± standard error for linear kinematic and pectoral fin muscle activity variables. Ctrl – control, Exer – terrestrial exercise, Terr – terrestrial acclimation.

Table S5.3. Polar distributions and descriptive statistics for kinematic and pectoral fin muscle activity variables.

		Ctrl				Exer				Terr			
			von Mises		Rayleigh's		von Mises		Rayleigh's		von Mises		Rayleigh's
Variable	Muscle	amean ± a.var	κ	p	p	amean ± a.var	κ	p		amean ± a.var	κ	p	
Max Nose Elevation (°)	-	66.5±8.6	6.9	>0.99	**<0.001**	56.7±8.0	7.3	>0.99	**<0.001**	68.8±4.0	13.8	>0.99	**<0.001**
Min Fin-midline Distance (°)	-	126.6±10.9	5.6	>0.99	**<0.001**	106.6±17.8	3.6	>0.99	**<0.001**	129.5±16.6	3.7	>0.99	**<0.001**
Start Slip (°)	-	41.2±8.6	7.0	>0.99	**<0.001**	33.8±7.4	8.0	0.10	**<0.001**	45.8±9.2	6.4	>0.99	**<0.001**
Start Tail Swing (°)	-	34.4±14.9	4.2	>0.99	**<0.001**	36.7±12.0	5.0	>0.99	**<0.001**	37.2±5.2	11.1	>0.99	**<0.001**
Pectoral Fin EMG Onset (°)	Abd	280.9±18.9	3.3	>0.99	**<0.001**	-	1.4	**0.02**	-	295.3±11.5	5.4	>0.99	**<0.001**
	Add	147.3±19.5	3.3	>0.99	**<0.001**	167.9±21.8	3.0	>0.99	**<0.001**	170.7±16.6	3.7	>0.99	**<0.001**
	Cmt	492.9±4.6	12.7	>0.99	**<0.001**	251.1±33.8	2.0	>0.99	**<0.001**	308.4±16.0	3.9	>0.99	**<0.001**
	Zpt	123.2±12.6	4.8	>0.99	**<0.001**	130.6±10.3	6.0	>0.99	**<0.001**	129.5±30.9	2.2	>0.99	**<0.001**
Pectoral Fin EMG Offset (°)	Abd	155.3±24.1	2.7	>0.99	**<0.001**	119.7±18.9	3.4	>0.99	**<0.001**	138.1±13.2	4.6	>0.99	**<0.001**
	Add	118.6±29.2	2.3	>0.99	**<0.001**	119.7±19.5	3.3	>0.99	**<0.001**	139.2±19.3	3.5	>0.99	**<0.001**
	Cmt	129.5±12.6	4.9	>0.99	**<0.001**	118.0±11.5	5.4	>0.99	**<0.001**	116.3±12.6	4.9	>0.99	**<0.001**
	Zpt	32.7±47.6	1.4	>0.99	**<0.001**	52.7±52.1	1.3	>0.99	**<0.001**	356.0±30.9	2.2	>0.99	**<0.001**

The von Mises statistics test for a deviation from a von Mises distribution. Rayleigh's test looks for unimodal deviations from a uniform circular distribution. Significant p-values are in bold. amean, angular mean; a.var, angular variance; Ctrl, control; Exer, terrestrial exercise; κ, kappa parameter of von Mises distribution; Terr, terrestrial acclimated fish.

Table S5.4. Red body muscle activity values during walking after exercise and terrestrial acclimation.

Variable	Position	Ctrl	Exer	Terr
Body EMG Duration (s)	e1	0.24±0.02	0.26±0.02	0.22±0.02
	e2	0.24±0.02	0.29±0.02	0.22±0.02
	e3	0.24±0.02	0.24±0.02	0.22±0.02
	e4	0.23±0.02	0.21±0.01	0.22±0.02
	e5	0.22±0.02	0.26±0.02	0.22±0.02
Body EMG Duty Factor (%)	e1	48.0±2.9	49.9±2.6	48.4±2.5
	e2	44.6±2.9	55.3±2.6	47.7±2.5
	e3	50.6±2.9	47.7±2.6	48.8±2.5
	e4	50.1±2.8	40.5±2.6	49.0±2.5
	e5	44.0±2.8	49.3±2.6	50.0±2.5
Body RIA (%)	e1	7.35±0.58	7.55±0.51	8.68±0.58
	e2	6.85±0.59	6.70±0.53	6.76±0.60
	e3	6.80±0.59	6.83±0.53	9.22±0.60
	e4	8.13±0.61	6.97±0.54	9.33±0.61
	e5	7.48±0.64	7.18±0.58	8.01±0.65

Estimated marginal mean ± standard error for linear kinematic and emg variables. Ctrl – control, Exer – terrestrial exercise, Terr – terrestrial acclimation.

Table S5.5. Red body muscle activity changes during walking after exercise and terrestrial acclimation.

	Treatment			Position			Treatment×Position				
Variable	F	df	p	F	df	p	F	df	p	R^2_m	R^2_c
Body EMG Duration (s)	2.0	2,10	0.187	1.2	4,793	0.305	8.3	8,793	**<0.001**	0.09	0.33
Body EMG Duty Factor (%)	0.1	2,10	0.87	3.0	4,596	**0.019**	6.6	8,596	**<0.001**	0.07	0.22
Body RIA (%)	1.6	2,10	0.25	3.4	4,793	**0.010**	4.4	8,793	**<0.001**	0.12	0.47

ANOVA-type summary statistics from linear mixed effects models for linear kinematics and muscle activity during walking. Note that all pairwise comparisons between treatments are not significant. R^2_m, marginal R-squared; R^2_c, conditional R-squared.

CHAPTER 6: SYNTHESIS AND CONCLUSION

Despite the importance of amphibious fishes for investigating plasticity and evolutionary processes associated with the transition from water to land (Damsgaard et al., 2020; Gordon, 1998; Graham and Lee, 2004; Lutek et al., 2022; Sayer, 2005; Turko et al., 2021; Wright and Turko, 2016), studies of the specific neuromuscular control mechanisms are rare (Foster et al., 2018; Horner and Jayne, 2014; Gillis, 2000; Perlman and Ashley-Ross, 2016). Using *Polypterus senegalus*, a fish that seldom leaves the water in the wild but has the capacity for terrestrial locomotion (Du et al., 2016), my thesis directly investigated how amphibious fish control locomotion across a variety of habitats. To this end, I used *P. senegalus* to address four primary objectives: (1) to explore the extent of neuromuscular plasticity across environmental gradients (viscosity and water depth), (2) to generate and test hypotheses about paramount signals for this neuromuscular plasticity, (3) to determine the neuromuscular underpinnings of locomotor transitions, and (4) to determine the neuromuscular control of developmental behavioural plasticity in novel environments. Collectively, these objectives tested the overarching hypothesis that sensory feedback and mechanical constraint modulate the basic swimming neuromuscular pattern to generate locomotion across a variety of novel environments in *P. senegalus*.

6.1 Neuromuscular plasticity

Throughout my thesis (Chapters 2, 3, 5) I utilized two environmental systems that alter the sensory feedback and mechanical constraint to which an animal is exposed: (1) viscous water and (2) terrestrial environments. Both systems increase the environmental resistance that an animal must overcome to generate movement. Animals that use body-powered swimming pass an anterior-to-posterior muscle activation wave along their bodies (fish - Altringham and Ellerby, 1999; Altringham and Shadwick, 2001; Coughlin, 2002; Shadwick and Gemballa, 2005; Shadwick et al., 1998; Syme, 2005; Wardle et al., 1995; salamanders - Delvolvé et al., 1997; Frolich and Biewener, 1992; terrestrial

locomotion in eels - Gillis, 2000). *Polypterus senegalus* also maintained an anterior-to-posterior wave of axial red muscle activity in water, high viscosity, and different water depths (Chapters 2, 3). This simple, and rather ubiquitous control pattern used across these environments appears to release novel or otherwise cryptic behavioural variation (i.e. walking in *P. senegalus*; Chapter 3). Thus, a single neuromuscular pattern may be effective for generating locomotion across a variety of environments.

Using a single neuromuscular pattern across a diversity of environments may also facilitate locomotor plasticity and minimize the costs of exploring novel environments. Generally, the costs of plasticity can be thought of as an energetic concern (Auld et al., 2010; Callahan et al., 2008; DeWitt et al., 1998; Murren et al., 2015; Piersma and van Gils, 2011; Relyea, 2002; van Buskik and Steiner, 2009). From a neuromuscular perspective, if the same set of neurons and muscles can generate movement across environments, this avoids the costs associated with building new neurons or maintaining neural circuits that are used only in select environments. Since an anterior-to-posterior wave of axial red muscle activity was found in novel environments across acute and chronic timescales, such use of a single neural circuit may be occurring in *P. senegalus* (Chapters 2, 3, 5). Theoretically, this would reduce the costs of exploring novel environments, reduce the costs of locomotor plasticity, and may reduce the barriers to exploring novel environments.

While the anterior-to-posterior wave of axial red muscle activity remained across environmental gradients, each environment caused unique modifications to axial muscle activity intensity, pectoral fin muscle activity, and resulting kinematics. For example, in high viscosity water, the intensity of *posterior* axial red muscles increased, while across water depths the intensity of *anterior* axial red muscles increased. These unique responses in different parts of the body suggest that different sensory signals are at play in these two environments. While the critical sensory systems in each environment remain to be determined (see 6.3 and 6.4 below for a more in-depth discussion), similar kinematic and muscle activity responses to high viscosity (Horner and Jayne, 2008) and water

depth (Pace and Gibb, 2011) in other elongate fish suggests that they may share common strategies for generating locomotion across environments.

In chapter 3, I found two pieces of evidence for a critical role of mechanical constraint during locomotion across water depths. I observed a stark increase in lateral undulation amplitude and pectoral fin range of motion in 0.9 body depths, but minimal changes in axial or pectoral fin muscle activity. I also observed two muscle activity patterns in the adductor of the pectoral fin in 0.7 body depths that resulted in a single kinematic output. Both outcomes highlight the ability of the environment to shape outward behaviour regardless of the underlying muscle activity. This chapter adds to the growing body of "neuromechanics" literature that emphasizes the role that the mechanics of the external environment and/or the physical constraints of the animal's morphology have in neuromuscular control (Nishikawa et al., 2007). My results are consistent with the theory that the surrounding environment may simplify locomotor control (Koditschek et al., 2004; Loeb et al., 1999; Ting and Macpherson, 2005) and suggest that mechanical constraint dictates axial and pectoral fin kinematics across water depths in *P. senegalus*.

The combined results of chapters 2, 3, and 5 form a foundational understanding of the role of sensory feedback and mechanical constraint in *P. senegalus* across high resistance environments. Both sensory feedback and mechanical constraint are evidently critical pieces of the locomotor control system, but their interplay and relative importance changes based on the physical environment through which the animal is moving. The results of my thesis have begun to tease apart the intimately linked pieces of the locomotor control system and have improved our understanding of the neuromuscular mechanisms that generate locomotion in *P. senegalus*.

6.2 Locomotor transitions in *P. senegalus*

Locomotor transitions are commonly defined based on discontinuities in kinematic metrics. Transitions occur between "gaits" and/or "modes". Gait is traditionally reserved for discontinuities

in kinematic pattern based on speed (e.g. Blaszczyk, 2001; Kendall et al., 2007; Mendes et al., 2013; Mussi et al., 2002), but recently has also been used in reference to kinematic discontinuities in response to environment (e.g. Berri et al., 2009; Standen et al., 2016). Locomotor mode is less explicitly defined, but generally refers to broader categories of locomotion (e.g. swim, fly, walk). Notably, neither term explicitly defines a role for neuromuscular control. This omission ignores the mechanistic underpinnings of locomotor transitions and, since kinematics can be shaped by environmental mechanics, obfuscates commonalities that may exist across locomotor transitions. Neuromuscular differences across locomotor transitions may be especially blurred when the same muscles are used across the transtion (e.g. some amphibious fishes on land). Here, I use "gait" to refer to discontinuities in kinematics based on speed and "mode" to refer to discontinuities based on environment, highlighting similarities in muscle activity across these transitions.

Prior to my thesis, evidence based on comparisons of behaviour in aquatic and terrestrial environments suggested that swimming and walking in *P. senegalus* were distinct modes generated by unique patterns of muscle activity (Foster et al., 2018; Standen et al., 2014). The results presented in my thesis challenge this notion and suggest that swimming and walking share muscle activity characteristics. In chapter 3, I investigated whether swimming and walking are unique modes by looking for changes in kinematics and muscle activity of the body and pectoral fins across water depths. I found that swimming and walking appear to be generated by a similar pattern of red axial muscle activity, while a discrete change in pectoral fin coordination exists when the fin is obligated to interact with the substrate. The independence of pectoral fin and body control is supported by evidence that the putative mesencephalic locomotor region in *P. senegalus* is responsible for adjusting the frequency of axial movements but does not elicit pectoral fin movements (Chapter 4). Because of the continuous characteristics of body muscle activity change across water depths and because the mesencephalic locomotor region appears to exclusively control body movements, I suggest that

locomotor transitions in *P. senegalus* are not discrete. Thus, a continuum of axial muscle activity and behaviour, augmented by "flexible" (i.e. with mutable neuromuscular control) pectoral fins, all of which is shaped by sensory feedback, appears to generate swimming, high viscosity swimming, the transition to walking and walking itself.

I propose three locomotor transitions across the spectrum of behaviour in *P. senegalus* (Chapter 4). Two of these transitions occur in water: (1) paired fin (slow speed) swimming transitions to body and paired fin (medium speed) swimming and (2) body and paired fin (medium speed) swimming transitions to body (fast speed) swimming. The third transition is from swimming to walking. Notably, body undulation amplitude increases in the following order: slow swimming, medium swimming, fast swimming, walking (K.L. pers obs; data in prep). While the transition from swimming to walking is difficult to directly study in *P. senegalus* – based on unsuccessful efforts in our lab during my PhD to convince *P. senegalus* to walk up a ramp out of the water – there is no reason to believe that this transition must be made from fast swimming directly to walking. However, the progressive increase in lateral undulation across these behaviours, higher muscle activity intensity during walking in *P. senegalus* (Foster et al., 2018), and evidence for similar increases in muscle activity with swimming speed in other species (e.g. Coughlin, 2002) suggests that these four behaviours could be part of an axial undulation continuum driven by the mesencephalic locomotor region.

In contrast, control of the pectoral fins seems to be independent of (or at least weakly coupled to) the body. To support this, I demonstrated that changes in pectoral fin muscle activity occur at different water depths than for body muscle activity (Chapter 3), the putative mesencephalic locomotor region does not appear to elicit pectoral fin movement (Chapter 4), and the pectoral fins can be used in a variety of ways in an aquatic setting (K.L. pers. obs.). Further, while steady swimming generally relies upon in-phase pectoral fin movements and walking relies upon out-of-

phase pectoral fin movements, these pectoral fin movement patterns are not environment exclusive (K.L. pers obs). Pectoral fins are often used out-of-phase during unsteady aquatic maneuvers (e.g. turning, escape, backwards swimming). Thus, I suggest that flexible pectoral fin control is overlaid on top of this axial continuum.

Changes in pectoral fin motion depending on swimming speed could be the result of neural thresholds. A biologically based numerical model suggests that saturation of limb central pattern generators above a threshold drive can generate limb folding in swimming salamanders (Ijspeert et al., 2007). Likewise in *P. senegalus*, neural thresholds could confine pectoral fin use to a particular set of swimming speeds. The transition to out-of-phase pectoral fin movements then may then be triggered by sensory feedback, potentially based on sufficient loading of the pectoral fin as likely occurs at water depths of 0.7 to 0.1 body depths. That loading (or physical forces) might be responsible for generating locomotor transitions is not unique to *P. senegalus*. Work with *C. elegans* (Berri et al., 2009) and horses (Farley and Taylor, 1991; although this view has been challenged recently - Robilliard et al., 2007) suggests that physical forces of the environment on the body and/or limbs can generate locomotor changes across environments (*C. elegans*) or speed (horses). A similar mechanism may be triggering the switch to out-of-phase pectoral fin movements during walking.

6.3 Sensory signals for locomotor plasticity and evolution

A core goal of my thesis was to determine the sensory signals that were of particular importance for the locomotion of *P. senegalus* across aquatic and terrestrial environments. This kind of detailed study in my thesis was limited for two main reasons: (1) our understanding of the kinematics and muscle activity in a variety of environments was limited to steady swimming (Foster et al., 2018; Standen et al., 2014), c-starts (Tytell and Lauder, 2002), and several terrestrial gaits (Standen et al., 2016), and (2) behaviour and muscle activity are the combined output of precisely

those mechanisms I sought to disentangle (i.e. central pattern generators, sensory feedback, and mechanical constraint). However, by careful selection of environmental gradients and using the comparative framework of kinematics and muscle activity outlined in chapter 1.4, I was able to contribute to the growing dataset that describes muscle activity and behaviour changes across environments and generate and test hypotheses about the type of sensory feedback that shapes neuromuscular control and behaviour in *P. senegalus*.

In chapter 3, I elected to include a finer gradient of water depths around 1.0 body depths because preliminary investigations found significant changes in behaviour within a narrow range (< 1.0 mm; approximately 10% body depth) of water depths. By focussing within this range of depths, I could examine how subtle changes in the balance of buoyant and frictional forces influence the neuromuscular and behavioural changes observed in *P. senegalus*. Since I observed similar muscle activity but different kinematics across these environments, a change in the loading of the ventral body on the substrate and/or exposure of the dorsal body is likely the cause of behavioural differences. Our full analysis (Chapter 3) also notes changes in pectoral fin muscle activity pattern at lower water depths that likely require further investigation, but regardless, work in chapter 3 highlights the importance of the relative magnitude of environmental forces for determining locomotor behaviour in *P. senegalus*.

In chapter 5, I further dissected the concept of body and fin loading using terrestrial acclimation and terrestrial exercise to test the developmental plasticity of *P. senegalus* in response to different loading regimes. Previous work in *P. senegalus* and other systems suggests that bone and muscle are plastic to changes in load (Du and Standen, 2017; 2020), but I was curious whether the type of loading altered the behavioural response. Specifically, I sought to isolate static and dynamic loading of tissues. I especially saw dynamic loading as potentially important both for plasticity and evolution because short forays onto land likely require fewer physiological adaptations to a terrestrial

environment than long-term stays. Given that *P. senegalus* displays activational plasticity in response to terrestrial exposure, if repeated terrestrial exercise alone was sufficient to recapitulate developmental plasticity in response to long-term terrestrial rearing, terrestrial exercise could provide a first step towards a more terrestrial life history without requiring extensive physiological changes. Notably, since no previous investigation had quantified the amount of movement *P. senegalus* perform when on land, I was unable to precisely match the level of movement between terrestrial acclimation and terrestrial exercise treatments; however, my results suggested that while *P. senegalus* are responsive to dynamic loading alone (terrestrial exercise), combined exposure to dynamic and static loading, as in the terrestrial acclimation treatment, generates larger neuromuscular and behavioural plasticity in the same amount of time (Chapter 5). This suggests that both static and dynamic loading, combined with other physiological changes, could be important for the transition from aquatic to terrestrial life history and may be one of a large suite of changes required for a potential positive feedback loop that could promote terrestrial existence (as has been demonstrated in *K. marmoratus*; Tigert et al., 2021 preprint).

6.4 Future directions

The results presented above have contributed to our understanding of locomotor plasticity in amphibious fishes, tested hypotheses about which sensory signals are critical for *activational* and *developmental* behavioural plasticity during the water-to-land transition, and developed two study systems of environmental gradients that can be used to further test hypotheses about the interaction of sensory feedback and mechanical constraint for motor control. Below, I will briefly discuss some potential future directions, and the implications and limitations of each approach. Broadly, these experiments look at directly testing the sensory signals hypothesized to be important for accommodating environmental change in chapters 2 and 3, dissecting the neural architecture of *P.*

senegalus, and using *P. senegalus* to further test the potential role of locomotor plasticity in the evolutionary transition from water to land.

Testing which sensory signals are important for observed locomotor plasticity requires isolating individual senses. While it is likely that a variety of senses are important for locomotor control across environments, blocking or altering senses alone or in combination can determine whether any senses are essential in specific environments. In chapter 2, I described the response of *P. senegalus* to viscous water. Viscous water presents an interesting study system because it exposes *P. senegalus* to more subtle changes in resistive forces than the direct transition to land. This system is also interesting in light of my results which suggest that locomotion in viscous water is essentially fast swimming. Because viscous water can be made with Newtonian properties (as in chapter 2), the physics of this system are much like scaling fast swimming to a more reasonable absolute speed, permitting repeated observation of *P. senegalus* swimming closer to their performance limit than is easily measurable in normal water. Pushing *P. senegalus* closer to their performance limit may exacerbate changes in motor control in response to the loss of a particular sense. I have since helped another graduate student in our lab test the importance of vision (by filming fish swimming in the dark) and the lateral line (by chemically blocking the lateral line) for swimming in *P. senegalus* using the viscous water system. The results suggest that neither of the senses is critical for swimming in high viscosity, but that interactive processing is occurring in higher brain centers. In addition to vision and lateral line, recent evidence (Picton et al., 2021) suggests that zebrafish have mechanosensitive cells on the periphery of their spinal cord. These cells are marked by the presence of connections between vertebrae that deform in response to lateral bending. Given that proprioceptive sensory feedback is another potential source of information while swimming in viscous water, it would be interesting to investigate whether *P. senegalus* has similar structures. A first step for checking if these structures are present would be looking at high resolution CT scans of *P.*

senegalus vertebrae for a similar structure and looking for differences in this structure (if present) between straight and bent fish. If this structure is present, it would be especially informative to record directly from efferent nerves at different levels of bending and/or to block these nerves during swimming in high viscosity water, though both neural preparations are technically demanding. Comparative studies of responses to viscous water across fish and/or other amphibians could help determine differences in neuromuscular organization and control between species. I have some preliminary data for viscous water swimming in zebrafish, goldfish, ropefish, and salamander that suggest there may be commonalities between fishes in response to viscous water, but that the salamander responds differently.

A main hypothesis that remains to be tested from chapter 3 is that the changes in axial muscle activity and kinematics in response to water depth are cased by mechanical loading of the body To test this hypothesis, and to further elucidate the mechanisms for transitioning between swimming and walking, would require measurement of force changes along the ventral surface of the body as water depth changes. This could be accomplished using an underwater force plate (*sensu* Biewener and Full, 1992). Measurements of the forces produced by *P. senegalus* in different depths of water would be informative for two reasons. First, this would quantify the magnitude of the force changes that elicit behavioural changes and permit experimental manipulations as detailed below. Second, this would provide a quantitative measure of the changes in physical environment that occurs across water depths and allow us to test whether there are discontinuities in the kinematics or muscle activity of *P. senegalus* along this scale (as in Berri et al., 2009). This experimental design should provide finer resolution of the force environment than the discrete changes in water depth I tested in chapter 3. It therefore allows for a more conclusive test of the hypotheses that: (1) axial muscle activity changes continuously along the environmental force continuum from aquatic to terrestrial, and (2) that the initial increase in body and fin kinematic magnitude is the result of

interactions between the body and the substrate. These force measurements could then be used to scale an experimental weighting of *P. senegalus* in an aquatic environment to isolate the loading of the body on the substrate from other potential sources of sensory feedback (e.g. the progressive emersion of the back) that occurred during the water depth experiments. Finally, since the results in chapter 3 suggest that a single axial control pattern could be common among elongate amphibious fishes, it would be interesting to conduct a comparative study across a broad phylogenetic sampling of the kinematic and muscle activity response to changes in water depth, ideally across an underwater force plate as outlined above. This would be a first test of whether a flexible axial neuromuscular pattern underlies both aquatic and terrestrial locomotion across fish species or whether this is a control scheme specific to *P. senegalus*.

Since the results presented in chapter 4 are entirely preliminary, this is one area rich for study. Performing repeated, successful stimulation of the putative mesencephalic locomotor region would generate an incredibly valuable tool for investigating the mechanistic basis of chapters 2 and 3 and, given the viability of my preparation described in chapter 2, ought to be achievable. I would start by getting a reasonable sample size across a range of stimulation frequencies and amplitudes as has been done in lamprey (Sirota et al., 2000) and salamander (Cabelguen et al., 2003). Once the basic response of *P. senegalus* to mesencephalic locomotor region stimulation had been determined, placing the preparation in different viscosities of water and on a terrestrial platform would test whether the responses I reported in chapters 2 and 3 are generated by feedback directly to spinal central pattern generators or if they first require integration in the brain. If these experiments were to confirm that the pectoral fin is not controlled by the mesencephalic locomotor region (as suggested in Chapter 4), a similar set of experiments to localize the pectoral fin control center would be incredibly informative.

Finally, I believe that expanding my work in chapter 5 holds immense potential to test questions about the requirements for developmental behavioural plasticity in *P. senegalus* exposed to a terrestrial environment. To better separate the role of static and dynamic loading, the experiment I performed in chapter 5 could be repeated, but with a closer matching of static and dynamic loading between treatments. Experiments could either use the average value I reported here, or the distance moved by fish in the terrestrial exercise could be directly matched to the distance moved by the terrestrial acclimated fish in their cohort. The latter could be facilitated by leveraging current advances in computer vision for tracking of animal movement (e.g. DeepLabCut, TRex). This type of experimental design would more concretely disentangle the influence of static and dynamic loading for generating neuromuscular and behavioural plasticity and shed light onto how the first steps on land might occur. The gold standard for demonstrating that this plasticity can drive evolutionary change would be multigenerational terrestrial rearing; however, due to the long generation time of *P. senegalus* and the difficulty of getting adult *P. senegalus* to breed, this is likely beyond our technical capacity at the moment.

During the course of collecting data for chapter 5, I also took the opportunity to (in collaboration with a post doc in our lab) collect data on the material properties of the body and pectoral fin of each fish in this experiment and to collect kinematic data across a range of swimming speeds for all fish in the experiment. The former dataset will allow us to determine how the material properties of the body and pectoral fins change following terrestrial acclimation and exercise and whether these changes might have influenced the kinematic changes observed. The latter dataset will allow me to quantify swimming gait transitions in *P. senegalus* and to test whether terrestrial acclimation or exercise affected swimming kinematics. Previous work suggests that there is no influence of terrestrial rearing on swimming (Standen et al., 2014), but that dataset was collected

only for slow speed swimming. It may be that trade-offs between swimming and walking performance appear only near performance limits.

6.5 Conclusions

Collectively, the results of my thesis suggest that the locomotor control of *P. senegalus* has swimming-like qualities across a range of aquatic and terrestrial environments. The flexibility of the anterior-posterior wave of muscle activity that typifies red axial muscle activity in all the environments I studied is augmented by pectoral fin control that is responsive to sensory feedback and can be shaped by the environment to generate effective locomotion on land. Further, based on similarities in axial muscle activity, I suggest that swimming and walking are part of a behavioural continuum, and that the intensity of muscle activity is modulated in an environment-dependent manner based on sensory feedback. I have also demonstrated that both terrestrial acclimation and terrestrial exercise can generate *developmental* behavioural plasticity. These behavioural changes are likely the result of subtle changes in pectoral fin activation that reduce friction between the body and the substrate to generate more effective walking. This work is some of the first to probe the mechanistic basis of neuromuscular plasticity in an amphibious fish and develops several study systems with which the intricacies of neuromuscular control and plasticity can be tested. Finally, the long-term terrestrial acclimation work (Chapter 5), in combination with the evidence supporting a continuum of axial muscle activity and kinematics dictated by the environment in *P. senegalus*, suggests that the difference between aquatic to terrestrial locomotion may not be that large. Modulation of a single axial pattern and neuromuscular flexibility of the pectoral fins appear to permit *P. senegalus* to successfully navigate a terrestrial environment and produce plastic responses to prolonged terrestrial exposure that improve walking behaviour. Given recent evidence that plasticity can become integrated into the genome, the results I present suggest that for amphibious fish, a life on land may only be a few steps away.

6.6 References

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