Behaviour and its consequences: *Xenopus laevis* wall following, swimming, and corollary discharge

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Dissertation der Graduate School of Systemic Neurosciences der Ludwig-Maximilians-Universität München

21 June 2017

First reviewer and supervisor: Prof. Hans Straka

Second reviewer: Dr. Florence Bareyre

Date of oral defense: 26 September 2017

Acknowledgements

While (hopefully!) finishing a PhD in four years might not be considered all that long, it is still four years of living in a foreign country (as my lab mates made fun of my Swiss German, I made sure to very quickly adjust to 'proper' German), four years of learning, studying, thinking, experimenting, failing, making friends, and generally living. A number of people have played major roles in these four years of my life, and they deserve heartfelt thanks. I am grateful…

To Hans, for being supportive, allowing me to follow side projects, and letting me attend many conferences and summer schools.

To my TAC, who helped me cruise along. Special thanks go to Ansgar for coming all the way from Cologne, and Florence for making time to be a reviewer.

To Boris, for reminding me that there is no need to make my life unnecessarily complicated (in his words: 'Giving up is always an option'), but for also helping me out in serious scientific endeavours.

To Francisco for always being in the lab when help was needed, for kind words after failed experiments, and of course for feeding and breeding our animals.

To all yet unmentioned members of the AG Straka who shared time with me in the lab – Roberto, Haike, Kathi, Céline, Johanna, Suzan, Elisabeth – for shared lunches, support, feedback and chats over coffee.

To Céline and Johanna for taking me bouldering (and Elisabeth for coming along).

To Céline for sharing the final stretches of the PhD.

To Haike for helpful criticism and being unafraid to ask questions.

To Elisabeth for support and feedback, including on this thesis.

To Alex K for help and discussion of programming challenges.

To Eli for excellent times cracking cases and speaking German at lunch.

To Alex C for proofreading blogposts and parts of this thesis.

To Alex, Delwen, Diana, Ella, Ela, Gregory and other 3rd floor people for uplifting chats over coffee.

To Hilde for being a lovely neighbour in the office.

To the GSN staff for being friendly and helpful when trying to navigate the administrative jungle that are German universities.

To Anna and Vero for being fabulous flatmates who could also be counted on to give scientific advice and commiseration after failed experiments.

To many GSN friends for providing a supportive network and a social life.

To the Lindy crew of the Boogie Bären for making me feel at home in Munich outside the university setting, and allowing me to teach Lindy Hop, which then spread a little into the GSN ☺.

To my parents and family for steadfast support.

To David, for always being there (even when you where not there).

Thank you!

Summary

In this thesis, I have examined the behaviour and some of its neural underpinnings of a 'model' animal, the tadpoles and froglets of *Xenopus laevis*, at different levels of description and detail. At a macroscopic level, I investigated the animals' movements in a very simple space. Zooming in, I looked at locomotion in freely and fictively swimming animals as well as at some of the sensory and motor consequences of locomotion. For many of these projects, I tested not only one particular developmental stage but a range of stages, allowing me to test for changes in behaviour with development.

Methodologically, I employed video tracking to quantify movements in space over a longer period of time, as well as at a higher temporal and spatial resolution for short periods to record head movements during swimming. Semi-intact *in vitro* preparations of tadpoles were used to examine fictive locomotion and its consequences using electrophysiological recordings of peripheral nerves.

Movements in space remained fairly similar over development, from small tadpoles to froglets, with all animals following the walls in a square environment, although the strength of wall following (WF) increased with growth. Tentacles, which are putatively mechanosensory appendages that large tadpoles temporarily possess, did not play any role for the strength of WF. WF was passive at all developmental stages, meaning that the animals never actively turned at a convex curvature to follow the wall, but instead went straight and left the wall. This implies that WF is unlikely to serve a defensive or spatial function.

Looking specifically at locomotion in tadpoles showed that these animals commonly swim at 20 - 40 mm/s forward speeds, and move their heads left to right at up to 2500°/s angular velocities. These velocities decrease with development, probably because swimming frequency also decreases, from about 8 to about 5 Hz. Developmentally appropriate swimming frequencies are also seen in fictive swimming when the animals are deprived of normal sensory feedback. The mechanisms behind the developmental decrease in swimming frequency remain to be elucidated; biomechanical factors might well play a role. The left-right head oscillations during swimming also represent vestibular self-stimulation, which reaches amplitudes that are much higher than any of the stimuli used in sensory vestibular experiments. Another consequence of locomotion was observed in large tadpoles with

tentacles: These tentacles are retracted during swimming, via a locomotor corollary discharge from the spinal cord.

What I have shown in this thesis is first, that navigational behaviour of *X. laevis* in a simple laboratory setting seems to be mainly driven and constrained by the environment. Second, I have quantified head movements during swimming and therefore vestibular reafference, and found a developmental decrease in the swimming frequency. Finally, I uncovered an unusual effect of locomotion, namely the retraction of the tentacles during swimming. Together, these studies deepen the understanding of behaviour and its consequences in *X. laevis*.

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1 Introduction

1.1 Locomotor behaviour

While Theodosius Dobzhansky famously stated that 'Nothing in biology makes sense except in the light of evolution' (Dobzhansky, 1973), I would argue that – at least for animals that move around in their environment – nothing in biology makes sense except in the light of behaviour (Hofmann et al., 2016). Behaviour is how animals interact with the world, how they act upon it and how they sense what is happening around them. Observing animal behaviour has a rich history, with ethologists such as von Frisch, Lorenz or Tinbergen carefully dissecting behaviour. Tinbergen proposed four different dimensions or levels at which animal behaviour can be characterised and which are all necessary for a full description of behaviour. First, causation describes the mechanism underlying the behaviour in question. Second, the survival value represents the usefulness of the behaviour in terms of evolutionary fitness. Third, the ontogeny of a particular behaviour illustrates how this behaviour develops with the growth of the animal. Fourth, one asks in evolutionary terms how this behaviour came about – this is where biologists ask 'why' questions. These four levels are complementary and might also be inter-related; for instance natural selection, i.e. the evolutionary level, might have acted on ontogenetic mechanisms to produce something novel.

In this behavioural framework, neuroscience is mostly concerned with the mechanistic level, as researchers try to figure out how the nervous system generates behaviour, and sometimes with ontogeny, when examining how the nervous system and its functions develop. Indeed, some neuroscientists go so far as to claim that the evolutionary reason to have a brain is to control movement (Daniel Wolpert's TED talk (Wolpert, 2011)). Surrounded by ever more sophisticated mechanistic explanations, neuroscientists sometimes lose sight of behaviour and its importance (Krakauer et al., 2017; Yong, 2017a), the most emergent of all emergent properties of the brain. In this thesis, I stayed close to behaviour by observing freely moving animals for several of the projects implemented and described here, while also thinking about the neural underpinnings.

1.1.1 Observing freely behaving animals

There is one inherent difficulty with behavioural experiments, which makes them both fiendishly complex and very attractive: The interpretation of the behaviour. What does the behaviour 'mean' to the animal? An animal might have some 'reasons' for doing something, but at whichever of Tinbergen's levels the solution might lie, these reasons can only be inferred from observing the animal's behaviour. What makes this tricky is that the indications for interpreting behaviour in one way or another are often indirect, and careful controls are needed to provide evidence for these interpretations. On the other hand, behaviour is attractive to study because one can see the whole animal in action and test how it acts on and reacts to the world. The more natural the environment in which an animal is observed, the more difficult it is to obtain the data and the more complex the analysis will be. Laboratory settings have the advantage that many factors can be controlled, but care needs to be taken that the enforced simplicity does not constrain the behaviour[1].

One of the simplest behavioural setups in the laboratory is the so-called open field (OF) test, which has been administered to rodents and many other animals. The animal is placed in a barren arena, which is usually square or circular, and its behaviour is observed for a certain amount of time, ranging from 2 min to several hours. Early studies using the OF largely relied on observer ratings, i.e. researcher-defined behaviours. More recent studies have used automated analysis generating animal-centred measures of behaviour (e.g. Lipkind et al., 2004), often relying on automatic tracking of the animal in space (Robie et al., 2017). Early studies of behaviour explicitly sought to infer the animal's motivation, goal, or indeed state of mind from the observed behaviour. Hall, for instance, used defecation of rodents in an OF arena as a measure of 'emotionality', and found that it correlates negatively with the amount of activity as measured by the number of subdivisions of the arena the animal entered (Hall, 1934, 1936). However, subsequent studies did not always confirm this negative correlation, and emotionality has been criticised, as it is unlikely to be uni-dimensional (Ramos and Mormède, 1998; Walsh and Cummins, 1976). What was described as emotionality early on was later shown to consist of different factors (Ossenkopp et al., 1994; Paulus and Geyer, 1993). The vague concept of emotionality was therefore largely abandoned, and other concepts have been used instead. Anxiety, for instance, has been examined extensively and appears to be related to wall following in the OF both in rats (Treit and Fundytus, 1988) and mice (Simon et al., 1994). Wall following has further been suggested to serve as a defensive strategy (Grossen and Kelley, 1972), which is supported by evidence that rodents increase

[1] Indeed, this is a lesson I learnt from the first manuscript presented in this thesis: even a seemingly simple setup as allowing an animal to freely swim in a square tank can constrain the behaviour – squares are, after all, very uncommon in nature!

their wall following in aversive situations (e.g. Bonsignore et al., 2008), although ecological factors can also play a role (Falkenberg and Clarke, 1998; Vasquez, 1996). Moreover, inferring 'cognitive' aspects such as spatial learning is challenging, as many non-cognitive factors can have an effect too (Wolfer et al., 1998). The plethora of potential factors affecting behaviour is what makes behaviour so difficult to interpret.

The OF and thigmotaxis behaviours are further complicated by somewhat confusing use of terminology in the literature, for instance by calling wall following thigmotaxis despite never measuring touch (e.g. Simon et al., 1994; Treit and Fundytus, 1988). To clarify, wall following (WF) describes the tendency of animals to follow vertical walls in their environment, without implying any mechanism or usefulness. A number of potential mechanisms might underlie wall following, and they are not mutually exclusive. Thigmotaxis, which can be defined as 'The movement of an organism either towards or away from the stimulus of physical contact' (https://en.wiktionary.org/wiki/thigmotaxis, 15 May 2017), is one potential mechanism. Thigmotaxis is often implicitly used for positive thigmotaxis, which describes the tendency of animals to approach objects that they touch. Centrophobism, or the fear of open spaces similar to agarophobia in humans, is another potential mechanism that can drive wall following. At a less mechanistic and more 'survival value' level of describing behaviour, one can describe WF in the context of potential functions it might serve, and assign it to particular strategies, which again are neither mutually exclusive nor exhaustive. Since it is probably more difficult for a predator to catch prey near a wall than in the open (Grossen and Kelley, 1972), WF can be a defensive strategy. Alternatively or in addition, WF can serve as a navigational strategy to learn about the spatial properties of the environment (Kallai et al., 2007; Teyke, 1989), especially if long-range senses such as vision are not available. Near-range senses such as touch or the lateral line of fish and amphibians may then be used, and in the former case, thigmotaxis would therefore be the mechanism underlying WF.

Different mechanisms and strategies associated with WF can be tested more directly by altering the simple layout of a standard OF arena. Changing the size of the environment allows testing for the mechanism underlying WF: If thigmotaxis was the main driving force behind WF, then no change in wall following would be expected with changes in the size of the environment, since the wall would be equally attractive independent of the size of the environment (Eilam et al., 2003). Changing the shape of the environment from concave to convex, either by adding convex curves to a concave environment or by using an hourglass-shaped arena, allows distinguishing active from passive WF (Creed and Miller, 1990). In active WF, the animal follows concave as well as convex walls; in the latter case, the animal has to 'actively' turn to follow the wall. If, on the other hand, the animal follows walls in a concave environment, but leaves the wall at convex curvatures, this behaviour is called

passive WF. The authors use 'pseudothigmotaxis' for passive, barrier-directed WF – in this case, WF is more a result of the shape of the environment (which most likely the experimenter chose) rather than an active choice of the animal.

In this thesis, I have characterised wall following behaviour of our model animal, *Xenopus laevis*. By using different developmental stages – from small tadpoles to young froglets – I was able to test for changes with development, both in terms of growth from small to large tadpoles as well as in terms of more drastic changes such as a switch in locomotor style as the animals turn into froglets during metamorphosis. I have recorded behaviour in a simple concave environment, in tanks of different sizes to change the scale of environment, and in a convex tank, to obtain more specific information to infer mechanisms and potential uses of WF across development in *X. laevis*. The results of these experiments are reported in chapter 2.

1.1.2 Locomotion vs. locomotor behaviour

As described above, interpreting animal behaviour is difficult. There are different levels of explanation (Tinbergen, 1963), which can all contribute to the animal's moment-to-moment behaviour. Moreover, there is a plethora of potential confounding factors, both in the field and in the laboratory, and even controlling as many factors as possible in the laboratory might influence the animal's behaviour, not least because the environment becomes more and more unnatural. Thereby the imposed simplicity of the laboratory environment might in turn constrain the animal's behaviour. The remainder of this thesis therefore dealt with a more specific and tractable behaviour, namely locomotion and its consequences. To clarify the terminology, locomotor behaviour describes an animal's behaviour in space, but this likely includes periods of being stationary or at rest as well as periods of locomotion. Locomotion specifically denotes those periods of activity during which the animal moves (Martin, 2003), and this movement is rhythmic, coordinated and largely driven by central pattern generators (CPGs, see 1.1.3). The degree of stereotypy and dependence on central, feedforward programming vs. sensory feedback are likely influenced by the context in which locomotion takes place; here, I focused on rhythmic, stereotypical locomotion.

In this thesis, I examined swimming in tadpoles of *X. laevis*, both in freely as well as fictively swimming animals (see below). Developmental changes of swimming and associated head movements during swimming were characterised at a high temporal resolution (results in chapter 3). I also investigated some consequences of swimming – head movements during swimming stimulate the animal's own balance system, and it turned out that during swimming the tadpoles move their tentacles (results in chapter 4). Before delving into the

sensory consequences of locomotion and explaining the concepts of corollary discharge and efference copy, I will introduce locomotion itself in some more detail.

1.1.3 (Fictive) locomotion

Stereotypic, CPG-driven locomotion consists of rhythmical movements that help propel an animal forward. Terrestrial animals might walk, trot, gallop, run or hop, while aquatic animals swim using axial or fin movements. All of these different types of locomotion are driven by what is known as a central pattern generator (CPG). Historically, antagonistic centres in the spinal cord were first described by Brown (Brown, 1911), who observed that rhythmic hindlimb movements can be produced in a deafferented and spinalised cat. He proposed that proprioceptive feedback can adjust this rhythm but it must be generated centrally, in the spinal cord, in the absence of feedback. The presence of a central oscillator driving the locomotor rhythm was later observed in deafferented locusts (Wilson 1961), which produce rhythmic wing-beat commands in the absence of any sensory feedback. More and more CPGs were identified, for instance in the swimming lamprey (Grillner, 2003; Grillner et al., 1991) or in the stomatogastric ganglion of the crab (Marder and Bucher, 2007). While the concept of central rhythm generation in absence of sensory feedback was controversial for a long time, it has now been widely accepted that the vast majority of rhythmic behaviours such as walking, running, swimming, breathing, or chewing are driven by CPGs (Marder and Calabrese, 1996).

The experimental observation of these rhythms at the neural level usually occurs in the absence of any overt movement. These are therefore 'fictive' behaviours, and in the case of locomotion this is called fictive locomotion. A number of these central pattern generators underlying (fictive) locomotion have been particularly well studied, such as walking in stick insects (Borgmann and Büschges, 2015), swimming in lampreys (Grillner, 2003), gastric movements in the crab (Marder and Bucher, 2007), or swimming in the tadpole (Roberts et al., 2010). Additionally, important insights have been gained from mice and rats (Kiehn, 2011, 2016), which have even been applied in robotics (Ijspeert, 2008). For instance, a biologically inspired CPG has controlled locomotion in a salamander-like robot which can both walk and swim (Crespi et al., 2013; Ijspeert et al., 2007).

The tadpole in particular is one of the best-understood CPGs in vertebrates. Hatchlings (developmental stage 37/38, see section 1.2.1) are capable of swimming, with free and fictive swimming having similar properties (Kahn et al., 1982). They can produce a fictive swimming rhythm in the absence of sensory feedback, with the appropriate left-right alternation and rostrocaudal delay (Kahn and Roberts, 1982). A central pattern generator is present on each side of the spinal cord; mutual inhibition is necessary for the appropriate left-

right alternation but not for the rhythm generation per se (Roberts et al., 1981). Recordings of motor neurons during fictive swimming helped characterise the excitatory and inhibitory inputs to these cells (Soffe and Roberts, 1982a, 1982b). Anatomically, 4 interneuron classes were identified in the spinal cord (Li et al., 2001) among a total of about 10 cell types (Roberts et al., 2012). No tonic descending excitation was found, but rhythmic descending excitation is present in reticulospinal neurons (Soffe et al., 2009). Excitatory interneurons are weakly electrically coupled (Li et al., 2009). Moreover, behavioural observations of swimming or stopping in response to touch can also be observed fictively (Buhl et al., 2012, 2015; Li et al., 2001). With metamorphosis, drastic changes occur in the locomotor style and the CPG driving locomotion (Beyeler et al., 2008; Combes et al., 2004; Rauscent et al., 2006, 2009). CPGs for both axial and leg-based swimming coexist at certain developmental stages (Combes et al., 2004).

The CPGs of these tadpoles have therefore been characterised in great detail, representing one of the best understood vertebrate examples of how neurons generate behaviour – in this case locomotion (Roberts et al., 2010).

1.1.4 Dealing with consequences of locomotion

During locomotion, but also during movements unrelated to locomotion, animals change the inputs to their sensory systems with their movements. For instance when walking forward, the visual scene shifts characteristically from front to back on either side, and the forward and up-and-down movements of the head stimulate the vestibular system. Moreover, the feet feel the touch on the ground, the proprioceptors signal changing positions of legs and body, and the ear registers the sound of the steps. Such sensory stimulation from self-generated movements poses two problems to sensory systems: First, how to discriminate self-generated from externally imposed movements, and second, how to still perceive the environment as stable despite the sensory feedback signalling movement. The nervous system has come up with a solution that works for both of these problems, namely that the motor system which directs, initiates or carries out the movement also informs the sensory systems which might be affected by the movement's consequences. This concept has been called 'efference copy' (EC) by von Holst and Mittelstaedt – implying that a copy of the motor command is sent to the sensory processing stages – or 'corollary discharge' (CD) by Sperry (von Holst and Mittelstaedt, 1950; Sperry, 1950; see Fig. 1). Incidentally, both papers were published in 1950, even though previous researchers going back as far as Aristotle have thought about the interactions of afference (sensory inputs) and efference (motor commands); later, Helmholtz, Mach and von Uexküll extended and conceptualised these ideas further (see Grüsser, 1986). Both 1950 papers examined behaviour of animals after experimentally manipulating the eye or head such that it was turned by 180° relative to its normal position. For both the fly, which

was examined by von Holst and Mittelstaedt, as well as the fish, which Sperry used, behaviour was impaired and circling was frequently observed. Any movement of the animal would lead to a shift of the image on the retina, and the animal would make compensatory movements to counteract this shift and stabilise the gaze. However, with the eyes turned by 180°, the animal made these corrective movements in the wrong direction and ended up circling. The authors therefore inferred that the animal expects a certain visual displacement to occur from its movements, and could not rely on the experimentally manipulated feedback. Such an expectation would arise from the motor system informing the sensory system about the movement, or to use Sperry's words: '… any excitation pattern that normally results in a movement on the retina may have a corollary discharge into the visual centers to compensate for the retinal displacement' (Sperry, 1950). CD therefore was proposed as a rather broad term, and is often still used as such (Poulet and Hedwig, 2007), whereas EC represents a copy of the motor command sent to some low-level sensory processing (see Fig. 1). However, the terms have often been used interchangeably in the literature, although an attempt at a 'taxonomy of corollary discharge' has been made (Crapse and Sommer, 2008). I will use the term CD in its broad sense.

Two more terms need to be introduced and explained here, which were coined by von Holst and Mittelstaedt: Exafference and reafference. These specify the sensory feedback that is caused by the animal itself as it moves (reafference) as opposed to the sensory inputs that are generated by changes in the world (exafference, see Fig. 1). Both of these are sensed by the same receptors and processed in the same channels, but can be distinguished with the help of CD.

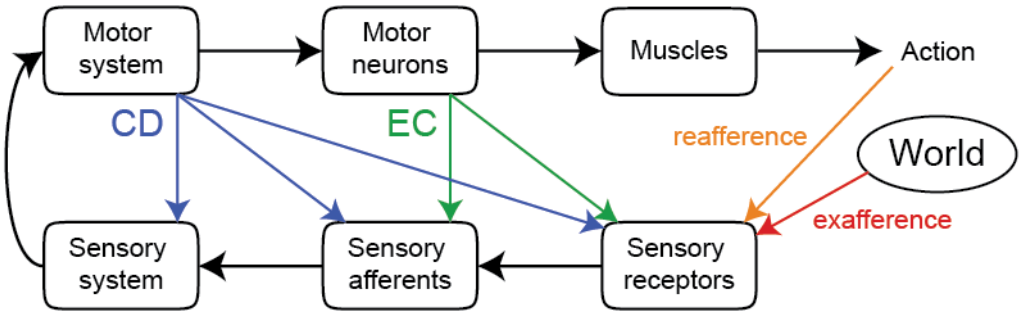

Figure 1. Scheme of sensory and motor processing including efference copy (EC) and corollary discharge (CD). The black arrows represent sensory and motor processing with information flowing from sensory receptors via sensory afferents to the sensory system (bottom), which relays the signal to the motor system. The motor system activates motor neurons that cause muscles to contract, which in turn lead to some action (top). Sensory input to the sensory receptors can result as a consequence of the animal's own actions (reafference, orange arrow) or as a consequence of some event outside the animal's influence (exafference, red arrow). EC informs peripheral levels of sensory processing about copies of motor commands (green arrows), whereas CD originates 'above' motor neurons in the motor system and can target any level of sensory processing (blue arrows). Scheme inspired by von Holst and Mittelstaedt (1950), Poulet and Hedwig (2007), and Crapse and Sommer (2008).

CD can act at a variety of different levels and have a number of different effects (Fig. 1, Crapse and Sommer, 2008; Poulet and Hedwig, 2007); a few illustrative examples will be introduced briefly. If the goal is simply to suppress reafference, it is likely very efficient for the CD to act directly in the periphery: Singing cicadas fold their tympanic membrane and reduce auditory sensitivity up to 20 dB (Hennig et al., 1994). Bats attenuate their reafference from self-generated echolocation calls by two mechanisms (Suga and Shimozawa, 1974): They contract their middle ear muscles to diminish the sound transmission to the inner ear using a CD (Suga and Jen, 1975) and additionally attenuate the auditory signal centrally (Suga and Schlegel, 1972), overall yielding an attenuation of 35 - 40 dB (Suga and Shimozawa, 1974). Humans similarly contract their middle ear muscles just before and during vocalisation (Borg and Allen Counter, 1989). Therefore, if you expect a loud sound, it might be advantageous to start humming to prevent damage to your hearing, because in response to loud sounds the middle ear muscles only contract about 200 ms after the sound started. Crickets similarly suppress reafference from self-generated sounds, both at the level of the primary and secondary afferent neuron (Poulet and Hedwig, 2002, 2003). This is one of the very few cases (all in invertebrates) where the identity of the corollary discharge interneuron has been clarified (Poulet and Hedwig, 2006). A similar suppression of reafference in the periphery occurs in the lateral line of swimming dogfish by a CD through the efferent system (Roberts and Russell, 1972; Russell and Roberts, 1974). The best studied example of CD is arguably the sense of electroreception in weakly electric fish: Not only have the different functional divisions of electroreception and their different CD effects been

described (Bell, 1989, 1981; Bell and Grant, 1989), but also the mechanisms underlying the plasticity of the CD in the cerebellum-like structure of these mormyrid fish are at least partially understood (Kennedy et al., 2014; Requarth and Sawtell, 2014; Warren and Sawtell, 2016).

Corollary discharges have also been described in the tadpoles of *Xenopus laevis*. Similar to the suppression of lateral line inputs in swimming dogfish, lateral line inputs in swimming tadpoles are partially suppressed by the efferent system (Chagnaud et al., 2015). Since these efferents innervate both the lateral line and the vestibular hair cells and afferents, some effects on the vestibular reafference have likewise been observed (see section 5.1.5). Gaze stabilisation during swimming in tadpoles is again achieved via a CD mechanism originating in the spinal cord (Combes et al., 2008; Lambert et al., 2012; von Uckermann et al., 2013). In *Xenopus* embryos, an additional effect has been described: The phasic gating of a reflex, which would normally cause swimming in response to touch, during swimming (Sillar and Roberts, 1988). The source of this phasic inhibition has been identified (Li et al., 2002), making it one of only a few vertebrate examples where the sources of CD are known at a cellular level. CD effects from locomotion therefore abound, and another example has been described in this thesis – a motor effect, whereby the tadpoles' tentacles are faithfully retracted during swimming (see chapter 4).

1.1.5 The vestibular system

In contrast to the gaze stabilisation based on CD described above, reflex-driven gaze stabilisation often relies on the vestibular system. Moreover, the vestibular system also senses the head movements that accompany the majority of locomotor movements in a variety of animals (Chagnaud et al., 2012). To understand the consequences of such head movements for sensation, it is necessary to know the system that senses these head movements, which in vertebrates is the vestibular system. Indeed, the entire circuitry from vestibular endorgan to eye muscles seems to be a chordate novelty (Straka et al., 2014), and has changed little within vertebrates, probably due to functional constraints (Fritzsch, 1998).

The vestibular system is basically an intertia sensor, encoding movement of the head in space (Angelaki and Cullen, 2008). The peripheral sensory structure in the inner ear contains sense organs which are sensitive to rotational and linear velocities/accelerations, and can therefore encode head movements in six dimensions. On each side, there are three semicircular canals (SCCs) and three otolith organs. The SCCs sense rotations in three dimensions; they are fluid-filled circular tubes, and the inertia of the fluid, which leads to movements of the fluid relative to the tube, provides the stimulus that is sensed. Their planes of maximal sensitivity are roughly aligned with the pulling planes of the eye muscles (Simpson and Graf, 1981).

Linear movements, on the other hand, are sensed by the otolith organs, consisting of the utricle, the saccule, and the lagena. In therian mammals, the utricle and the saccule are the organs responsible for sensing horizontal and vertical translations, respectively. In frogs, on the other hand, the saccule primarily senses sounds and substrate vibrations (Lewis et al., 1982), making the utricle and the lagena the main linear sensors in frogs (e.g. Straka et al., 2002).

Sensory transduction in both linear and rotational sensors occurs in hair cells as the overlying fluid or membrane moves relative to the hair cells. This bends the hair cells' stereocilia, opening ion channels that change the membrane potential of the hair cell (Hudspeth, 2005). This in turn leads to more or less vesicles being released at the synapse to the primary afferent neuron, which then carries the signal to the brain via the 8th cranial nerve. Since hair cell deflections can occur both towards or away from the largest stereocilia, and deflections in the two directions are encoded as hyper- or depolarisations, movements in both directions can be sensed by a single organ, and are transmitted to the brain as changes in firing rate around a resting rate, which depends on spontaneous vesicle release from hair cells (see Eatock and Songer, 2011). The two vestibular endorgans on either side of the animal are arranged as mirror images of each other. This leads to a push-pull organisation of the SCCs, such that when one side is excited by a head movement, the other is inhibited (see Straka and Dieringer, 2004).

The vestibular system has often been examined in the context of the vestibulo-ocular reflex (VOR). This reflex serves to stabilize the animal's gaze in response to externally caused head movements, such that the eyes are moved in the exact opposite of the head. Functionally, the circuitry underlying the VOR is thought to be organised in frequency-tuned pathways (Straka et al., 2009), with populations of neurons from the afferents to the motor neurons differing in their dynamic properties (Beraneck et al., 2007; Dietrich et al., 2017; Pfanzelt et al., 2008; Straka et al., 2005). The vestibular system also mediates postural reflexes and balance control via vestibulospinal connections.

Tadpoles of *X. laevis* have functional and easily accessible vestibular endorgans from an early age, making them an attractive model for vestibular research (Straka and Simmers, 2012). A number of studies has taken advantage of this and examined vestibular afferent (Gensberger et al., 2016) and extraocular motoneuronal responses (Dietrich et al., 2017) in some detail. However, the nature and amplitude of the stimuli that the animals inflict on themselves with their own movements have not yet been characterised. It is important to know how the stimuli commonly used in vestibular research compare to self-generated stimuli, not least for judging how self-generated and external stimuli are processed differently (see 5.1.5.1 for the vestibular system in particular). I therefore set out to measure the animals' head movements – a sensory consequence of locomotion – during swimming (results see

chapter 3). Moreover, these sensory consequences of locomotion likely change with growth, as the animals' biomechanics are altered. In the vestibular system, the encoding of stimuli themselves changes with growth, since the dimensions of the endorgans affect their sensitivity (Muller, 1999). I therefore measured head movements in tadpoles of different sizes to see whether there was an interaction between growth of the animal and their self-generated vestibular stimuli (see chapter 3).

1.2 *Xenopus laevis*: A widely studied animal

Xenopus laevis has been used as a model organism in biology for many decades (Wallingford et al., 2010). While it does have some tangible advantages, such as the large number and size of eggs, year-round breeding with the help of hormonal injections, relatively fast generation time for amphibians, ease of maintenance due to its aquatic lifestyle as an adult, and robustness and resistance to disease, its spread into laboratories worldwide has also been influenced by a number of historical coincidences (Gurdon and Hopwood, 2000)[2]. *X. laevis* is a native South African species, with its tadpoles living in stationary puddles and probably rather murky water (Nieuwkoop and Faber, 1956). The adults are usually described as obligately aquatic, though anecdotes suggest they can briefly leave the water as well. The use of *Xenopus* for pregnancy testing helped to spread these animals into laboratories worldwide (see Gurdon and Hopwood, 2000). Developmental biologists then realised the potential of using *X. laevis* for their purposes, and made use of the large and readily available eggs, establishing *X. laevis* as a model for vertebrate development (Müller and Grossniklaus, 2010). Some of this early work – confirming that differentiated somatic cells still have a complete and functional set of genes – led to a Nobel Prize for John Gurdon (Gurdon, 2009, 2013). Molecular, biochemical and genetic studies then followed suit (Harland and Grainger, 2011). The one disadvantage of *X. laevis* is its tetraploidy – it is therefore not very well suited to mutation studies (Harland and Grainger, 2011). Its close relative *Xenopus tropicalis* stepped in here (Amaya et al., 1998) – it is diploid and its genome has been sequenced (Hellsten et al., 2010). However, its eggs are smaller, and *X. laevis* will therefore likely still be used for studies requiring micromanipulation; moreover, the genome of *X. laevis* has been sequenced recently (Session et al., 2016). While the use of other model animals such as *Drosophila*, zebrafish and mice, for which a plethora of genetic tools already exists will likely increase, both *Xenopus* species will still be employed in the 21st century (Beck and Slack, 2001), covering certain experimental niches. Supported by a database especially for

[2] Some of these coincidences have also been described in a very readable science article by Ed Yong in The Atlantic (Yong, 2017b).

Xenopus genetics and genomics (Bowes et al., 2007), *X. laevis* serves as a model for a variety of questions regarding development (e.g. McFarlane and Lom, 2012; Roberts et al., 2012), regeneration (e.g. Beck et al., 2009; Lee-Liu et al., 2016), as well as developmental studies of neural circuitry and sensorimotor interactions (Straka and Simmers, 2012).

1.2.1 Development of *Xenopus laevis*

Eggs of *X. laevis* are large and have been used extensively for micromanipulation experiments. The eggs develop and the tadpoles hatch after about two days at room temperature (Nieuwkoop and Faber, 1956). The development has been categorised into developmental stages based on internally or externally discriminable morphological characteristics (Nieuwkoop and Faber, 1956; see Figure 2 for an illustration of the developmental stages used in this thesis). When the tadpoles hatch at stage 37/38 two days after fertilisation, they are about 6 mm long (Nieuwkoop and Faber, 1956), and preferably attach themselves to the substrate via mucus secreted from their cement gland (Boothby and Roberts, 1992; Jamieson and Roberts, 2000). When they become dislodged, they swim until they encounter an obstacle and reattach themselves (Boothby and Roberts, 1992). This behaviour disappears as the tadpoles start feeding themselves around stage 45; this is also termed the free swimming stage (Nieuwkoop and Faber, 1956). The propensity to swim increases (Currie et al., 2016) and the animals start to filter-feed (Nieuwkoop and Faber, 1956). The vestibular end-organs are functional as soon as or briefly after the onset of free swimming (Horn et al., 1986; Lambert et al., 2008), and these are the youngest animals employed in this thesis (see Fig. 2). Until metamorphosis, the animals grow in size more than twofold, develop hindlimbs and then forelimbs, and grow and lose tentacles (Fig. 2). The globose shape of the tadpoles appears disadvantageous for swimming, but allows the hindlimbs to grow without compromising locomotor performance (Hoff and Wassersug, 1986; Wassersug, 1989). As the hindlimbs develop, so does the circuitry controlling their movements, such that the two types of locomotion – undulatory swimming and leg-based kicking – and the accompanying neural circuitries exist side by side before metamorphic climax (Combes et al., 2004). With the gradual shrinkage and final loss of the tail at the climax of metamorphosis, the change in locomotor style from undulatory tail-based swimming to leg-kicking becomes irreversible, but unlike the vast majority of amphibians, this change is not accompanied by a change in habitat – adult *X. laevis* are also aquatic (Nieuwkoop and Faber, 1956).

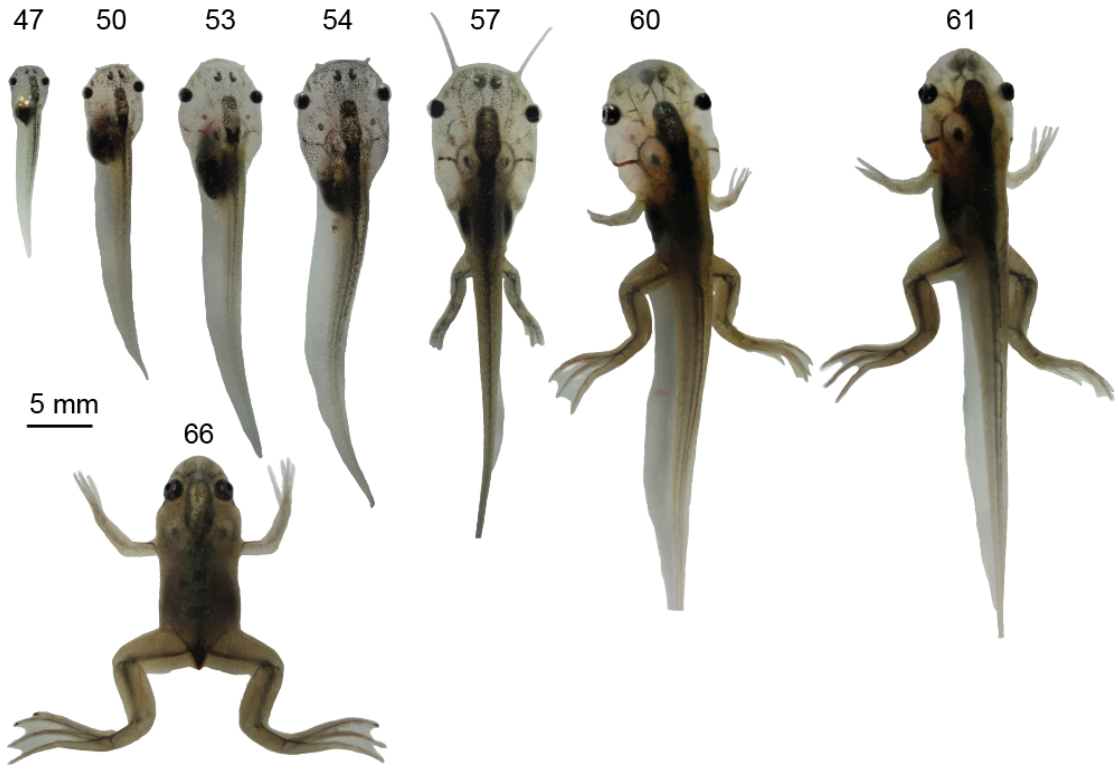

Figure 2. The development of *Xenopus laevis*. The numbers indicate the developmental stages according to Nieuwkoop and Faber (1956). The images are taken from Hänzi and Straka (2016).

The development of *X. laevis* allows researchers to study an interesting question: How does the animal's behaviour change with growth and with the fundamental reorganisation during metamorphosis? In this thesis, I examined developmental changes in behaviour from small tadpoles to young froglets. In particular, in experiments resembling open field tests for rodents, I tested the degree of wall following and indirectly investigated navigational strategies in all of these animals (results in chapter 2). Since the locomotor styles are widely different between tadpoles and froglets, I expected that their navigational strategies would be different too. For the tadpoles, which move their head left-right during axial swimming, I measured the kinematics of these head movements as the animals freely swam in the centre of a shallow tank (results in chapter 3). I also approached this from a developmental point of view by comparing head movements in tadpoles of different sizes. The change in biomechanics with growth might well lead to changes in swimming kinematics. With the swimming-related head movements, the animals stimulate their own vestibular system, so from the point of view of vestibular researchers, it is important to know what kind of stimuli the animals present to themselves. That way we would later be able to compare these self-generated stimuli to the external vestibular stimuli commonly applied in experiments, and potentially mimic the natural stimuli to compare responses to self-generated and external stimuli directly. As a first step towards this goal, I measured the rotational head movements

in tadpoles, from small tadpoles who had only just started free swimming (stage 46) to tadpoles shortly before metamorphic climax (stage 56; results in chapter 3).

Another curious feature of the development of *X. laevis* tadpoles are their tentacles (see the stage 57 animal in Fig. 2). These tentacles are present as small buds already before stage 50, and then grow in length, reaching up to 20% of their body length (or slightly larger than 1 cm)[3]. These appendages consist of a central rod of cartilage, surrounded by skin (Ovalle et al., 1998), containing Merkel cells (Nurse et al., 1983; Ovalle, 1979) and some putatively sensory innervation (Ovalle et al., 1998). The tentacles disappear again, slightly before the tail is resorbed. They first become floppy as the cartilage is resorbed, and finally the skin disappears, such that no traces of the tentacles can be seen in froglets.

Unlike many fish barbels (Fox, 1999), no gustatory receptors have been found in ultrastructural studies of *X. laevis* tentacles; only Merkel cells have been described (Eglmeier, 1987; Ovalle, 1979). I therefore wondered whether this putatively mechanosensory organ would serve some specific function, and was perhaps in some ways similar to rodent whiskers. Rodents use their whiskers extensively to gain information about their environment from whisker touch, and tend to touch the wall with their whiskers as they walk or run along it (Hartmann, 2011). The behavioural assay set up to test wall following and navigational strategies (results in chapter 2) was therefore also a test of whether only animals with tentacles follow the walls. The approach of using small tadpoles to froglets allowed us to test this developmentally, by comparing the behaviour of animals that do not yet have tentacles or do not have tentacles anymore with animals that have tentacles. Moreover, our breeding, for unknown reasons, produced many animals that completely failed to develop tentacles, but otherwise behaved normally (see the stage 54 animal in Fig. 2). This allowed us to directly compare the behaviour of animals with tentacles to animals without tentacles at the same developmental stages.

In contrast to rodents, which actively move their whiskers forwards and backwards to explore objects (Berg and Kleinfeld, 2003), tadpoles do not move their tentacles as they come into contact with an object. The muscular control is much simpler, consisting of only one muscle whose contraction retracts the tentacle, while protraction is passive and probably mediated by a cartilaginous spring at the base of the tentacle (Ovalle et al., 1998). In fact, tadpoles only seem to move their tentacles during swimming. In the light of the corollary discharges (CD) mediating compensatory eye movements in these tadpoles during swimming (Lambert et al., 2012; von Uckermann et al., 2013), I wondered whether a similar CD effect would be at play here, and therefore characterised tentacle movements and motor commands for tentacle

[3] Although in the literature, much larger tentacles of 2.5 - 4 cm have been reported (Ovalle et al., 1998).

movements (i.e. fictive tentacle movements) in fictively swimming tadpoles (results in chapter 4). For recording fictive swimming and tentacle movements, I employed an *in vitro* preparation of *X. laevis*, which will be described below.

1.2.2 *In vitro* preparation of *Xenopus laevis*

Recording electrophysiologically from nerves and neurons is sometimes feasible when the animal moves, but is much easier when it is stationary. We have therefore employed a semi-intact *in vitro* preparation of *X. laevis*. Isolated frog central nervous systems have been used for decades (e.g. Hackett, 1972), and a variety of preparations with different degrees of similarity to *in vivo* or *in vitro* have been used (see Straka and Simmers, 2012). Here, a preparation which in terms of behaviour is rather similar to the *in vivo* animal was employed. Briefly, after anaesthetising the animal, its lower jaw, visceral organs and its forebrain were removed (for more detailed methods see e.g. Hänzi et al., 2015). A similar preparation can survive in Ringer solution for several days (Straka and Dieringer, 1993), allowing plenty of time for recordings. This preparation has been used in a number of studies to record from vestibular afferents or oculomotor nerves while stimulating the vestibular system, either naturally or galvanically. It has the advantage that all main afferent and efferent connections are still intact, allowing eye movement recordings, electrophysiolocigal recordings in the periphery of entire nerves, calcium imaging in the central nervous system after application of a calcium indicator, or pharmacological and surgical manipulations. Recent papers have for instance described the plasticity of the vestibulo-ocular reflex (VOR; Dietrich et al., 2016), functionally characterised the abducens motor neuron population (Dietrich et al., 2017), examined responses to galvanic stimulation (Gensberger et al., 2016), or characterised how the vestibular reflexes in response to translation are tuned by semicircular canal inputs (Branoner and Straka, 2015). Additionally, optokinetic reflexes have been characterised in response to a number of different visual stimuli (Gravot, Knorr, Glasauer and Straka, in preparation).

Furthermore, fictive swimming can also be measured from electrophysiological recordings of ventral root activity after isolating the rostral part of the spinal cord (see Fig. 3). Regular, rhythmic, left-right alternating activity indicates that the animal is fictively swimming (Combes et al., 2004). Using such an *in vitro* preparation of *X. laevis*, these authors have characterised the transition from axial-based to leg-based swimming with metamorphosis, and found that these two central pattern generators co-exist at certain developmental stages (Combes et al., 2004). Neuromodulatory effects on swimming can likewise be examined in such a preparation (Currie et al., 2016; Rauscent et al., 2006, 2009).

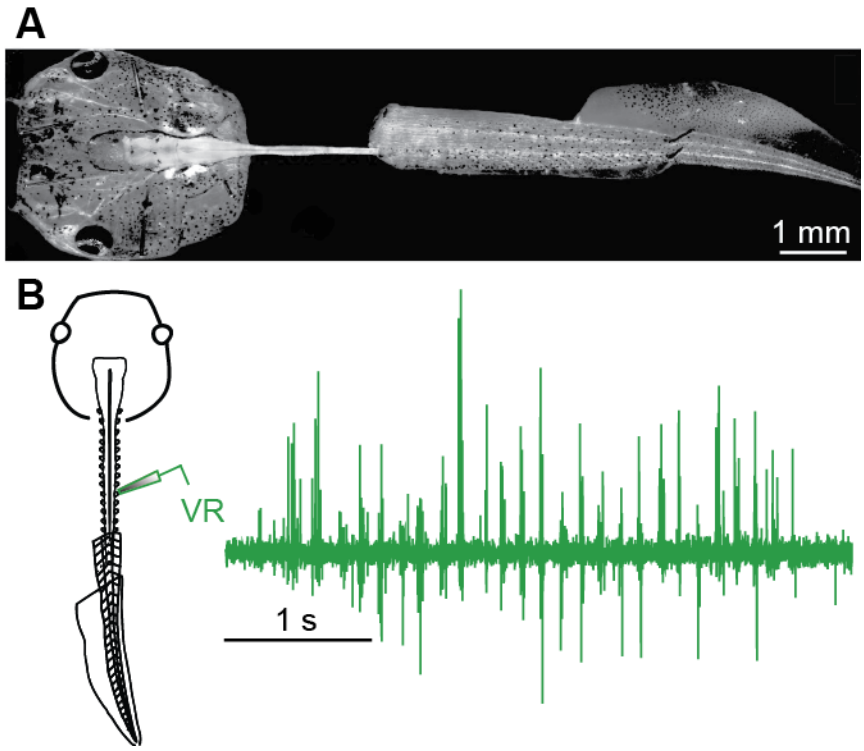

Figure 3. Recording fictive swimming in a semi-intact *in vitro* preparation of *Xenopus laevis*. A) Picture of a semi-intact *in vitro* preparation with the spinal cord isolated for recording ventral root activity. Modified and reused with permission from Banchi (2015). B) Schematic of a ventral root (VR) recording, with an example of fictive swimming from a stage 50 tadpole to the right.

I have used such a fictively swimming preparation to examine the swimming rhythm of *Xenopus* tadpoles, and how this changes with development (see results in chapter 3). Moreover, this preparation lends itself to examining the consequences of (fictive) locomotion that are driven by CD. Since the animal is not moving forward, much of the sensory feedback that is normally present during free swimming is absent. Additionally, sensory feedback can be selectively abolished by cutting the associated sensory nerve(s), allowing a very direct test of whether the observed effect is driven by sensory feedback or in a feedforward manner by CD. Previous studies have already shown that compensatory eye movements during swimming in tadpoles of *X. laevis* are driven by CD rather than by sensory feedback (Combes et al., 2008; Lambert et al., 2012; von Uckermann et al., 2013). Careful lesion experiments allowed the authors to determine the pathway the CD takes from the spinal cord to the hindbrain (Lambert et al., 2012). These compensatory eye movements also change appropriately over metamorphosis to accommodate the change in locomotor style; to be correctly compensatory, eye movements need to change from bilaterally coupled left-right to vergence movements (von Uckermann et al., 2013). More recently, the processing of the sensory consequences of locomotion was examined: Reafferent stimulation from the lateral line and the vestibular system are both reduced (though not abolished) by efferent activity

during swimming (Chagnaud et al., 2015). Adding to this list of CDs and effects of locomotor CDs already known from this preparation, I have similarly taken advantage of the *X. laevis in vitro* preparation when examining the movements of the tadpoles' tentacles (see chapter 4).

The following chapters contain the publications of original research carried out during this thesis, on movements in space (chapter 2; this manuscript is still under peer review), head movements during locomotion (chapter 3, published in the Journal of Experimental Biology) and tentacle movements during locomotion (chapter 4, also published in the Journal of Experimental Biology). The discussion in chapter 5 will then bring all of these aspects of behaviour and locomotion together, and put them in context of what has been described for tadpoles and other species in the literature.

2 Movements in space

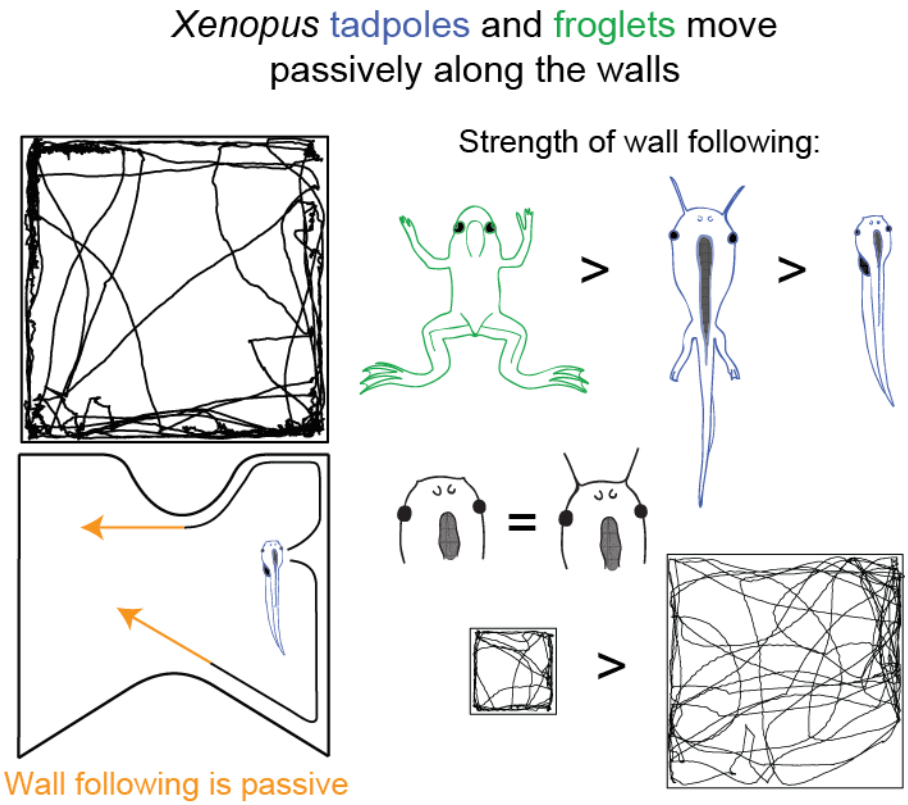

Figure 4. Graphical abstract for the study on wall following. Animal schemes modified from Hänzi and Straka (2016b), trajectories from Hänzi and Straka (2017a).

2.1 Citation

This manuscript is currently under review. The version presented here is available as a preprint on bioRxiv; the contents have been rearranged to make it more readable.

Hänzi, S., and Straka, H. (2017a). Wall following in *Xenopus laevis* is passive. bioRxiv 127258.

2.2 Contributions

Investigation, Software, Visualization: S.H.; Supervision, Project administration, Funding acquisition: H.S.; Conceptualization, Writing: S.H. and H.S.

Wall following in *Xenopus laevis* is passive

Running title: Wall following in *Xenopus laevis*

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Key words: locomotion, wall following, *Xenopus laevis*, thigmotaxis, centrophobism, swimming

Summary statement: *Xenopus laevis* tadpoles and froglets tend to swim along the walls of a square tank; but this wall following is passive – in a convex tank, they leave the wall.

Abstract

The tendency of animals to follow boundaries within their environment can serve as a strategy for spatial learning or defence. We examined whether animals of *Xenopus laevis* employ such a strategy by characterizing their swimming behaviour. We also investigated potential developmental changes, the influence of tentacles, which some of the developmental stages possess, and whether wall-following is active (animals seek out wall contact) or passive. Animals' swimming movements were recorded with a camera from above in a square tank with shallow water and their trajectories were analysed especially for proximity to the nearest wall. With the exception of young larvae, in which wall following was less strong, the vast majority of animals – tadpoles and froglets – spent more time near the wall than what would be expected from the proportion of the area near the wall. The total distance covered was not a confounding factor. Wall following was also not influenced by whether the surrounding of the tank was black or white, illuminated by infrared light, or by the presence or absence of tentacles. Animals were stronger wall followers in smaller tanks. When given a choice in a convex tank to swim straight and leave the wall or turn to follow the wall, the animals consistently left the wall, indicating that wall following in *Xenopus laevis* is passive. This implies that wall following behaviour in *Xenopus* derives from constraints imposed by the environment (or the experimenter) and is unlikely a strategy for spatial learning or safety-seeking.

Introduction

The exploratory behaviour of animals in unfamiliar environments is often characterized by a tendency to follow walls or distinct borders. Such wall following has been described in mice and rats (Simon et al., 1994; Treit and Fundytus, 1988), where it is often experimentally used as a readout for the level of the animal´s anxiety (Prut and Belzung, 2003; Walsh and Cummins, 1976). Well-studied examples of wall following also include fruit flies and blind cavefish (Besson and Martin, 2005; Goetz and Biesinger, 1985; Liu et al., 2007; Teyke, 1989).

Different potential functions have been ascribed to wall following behaviours. In some cases, wall following might be a defensive strategy; for instance avian predators likely have more difficulties catching e.g. a rat when the latter is moving along a wall compared to when it is moving across an open field (Grossen and Kelley, 1972). This explanation is supported by the fact that rats increase wall following in aversive situations (Grossen and Kelley, 1972), and thus legitimate the use of wall following in rodents as an indicator of anxiety (Gentsch et al., 1987; Simon et al., 1994; Treit and Fundytus, 1988). On the other hand, wall following can also serve as a strategy to learn the spatial setting of an environment. Blind cavefish, which live in dark caves without vision, explore unfamiliar environments by swimming along vertical borders and thereby memorize the layout of the surrounding (Teyke, 1989). A similar spatial learning has also been described in crayfish (Basil and Sandeman, 1999) and humans (Kallai et al., 2005; Kallai et al., 2007), suggesting that wall following is widely used for spatial orientation in vertebrates as well as invertebrates.

However, wall following does not necessarily imply that animals use this behaviour explicitly as a defensive or exploratory strategy. In particular, simply observing a freely moving animal in a concave tank does not indicate whether the animal actively seeks the proximity to the wall. A convex tank, on the other hand, can be used to clearly distinguish between active and passive wall following (Creed and Miller, 1990). The convex curvature allows the animal to chose to either continue straight and leave the wall, or to turn and follow the wall; the latter is then termed active wall following. Accordingly, an animal might appear to be a strong wall follower in a square tank simply because it has no option to make large turns and therefore continues to pursue the border (Creed and Miller, 1990).

To determine whether larvae and adults of the amphibian *Xenopus laevis* tend to swim along the walls of a tank, we quantified the swimming behaviour of these animals in a square concave tank. In addition, tadpole locomotion was recorded in square tanks of different sizes to assess the influence of the size of the environment. Animals at different developmental stages – from small tadpoles (stage 46) to froglets – were employed to estimate the effect of different locomotor styles as well as the role of mechanoreceptive tentacles, which are transiently present at mid-larval stages, in wall following. Finally, a convex tank allowed discriminating between active and passive wall following.

Materials and methods

Animals

Experiments were performed on tadpoles and froglets of the South African clawed toad *Xenopus laevis* (*n* = 92) of either sex at developmental stage 46 to 66 (according to Nieuwkoop and Faber, 1956). Stages were identified based on morphological features in freely moving animals in a petri dish under a dissection microscope. All animals were obtained from in-house breeding at the Biocenter of the Ludwig-Maximilians-University Munich, where animals were kept in aerated tanks at 17°C on a 12:12 hour light:dark cycle. All behavioural observations complied with the "Principles of animal care", publication No. 86-23, revised 1985 of the National Institute of Health. Permission for experiments subjected to approval was granted by the Regierung von Oberbayern (55.2-1-54-2532.3-59-12).

Image data acquisition - hardware and software

Image data were acquired with two different monochrome cameras from Point Grey (Richmond, Canada; now FLIR Integrated Imaging Solutions) and Point Grey image acquisition software (Fly Capture). The camera was placed in the centre above the tank to record the animal's movements in the horizontal plane. Videos obtained earlier in the course of the study were acquired using a Grasshopper Firewire camera (GRAS-03K2M-C) with a 640 x 480 resolution at 15 frames per second (fps). These videos were saved as JPG-compressed AVI files. Videos obtained later in the course of the study were acquired using a Grasshopper3 USB camera (GS3-U3-23S6M-C) with a maximum resolution of 1200 x 1200 pixels. The resolution was adjusted depending on animal and tank size and varied from 600 x 600 to 1200 x 1200 pixels with a frame rate of either 15 or 30 fps. Acquired images were saved as LZW-compressed TIFF files. All image data was visually inspected in FIJI (Schindelin et al., 2012; Schindelin et al., 2015), which was also used to create overlays. Further data analysis was performed using Python 3 (Python Software Foundation, https://www.python.org/, see below for details).

Image data acquisition - experimental conditions

Standard procedure. One animal at a time was observed in a 19 x 19 cm Plexiglas tank with a water level of 0.5 to 1.4 cm (0.5 cm only for the smallest animals, otherwise 1.2 - 1.4 cm) at room temperature (20 - 24°C). The vertical walls (20 cm high) of the tank were surrounded on the outer surface by white paper, and the tank was lit from below with four cold light sources placed on either side (ZLED CLS6000, ZETT OPTICS GmbH, Germany) or with a light box (Kaiser slimlite LED, Kaiser Fototechnik, Buchen, Germany) that created an evenly lit area of 46.0 x 20.5 cm. After 1 min adaptation to the environment, a 10 min video sequence was recorded for each of the 92 animals.

Tank size. In addition to the recordings of swimming behaviour in the 19 x 19 cm tank, a group of animals (*n* = 9, developmental stages 47 - 50) was also tested in a smaller square tank with floor dimensions of 7 x 7 cm. Animals were filmed for 10 min in each tank; the order of the tank sizes was small first for half of the tested animals, and large first for the other half. All images were acquired with the Point Grey Grasshopper3 camera at

15 fps.

Alterations in the illumination. To test for a potential influence of vision, a group of animals (n = 10, developmental stages 50 - 65) were filmed successively with both a white and a black paper surrounding the tank, for 10 min each, at a frame rate of 15 fps with the Grasshopper3 camera. The order of black/white was white first for five animals and black first for the other five. A separate group of animals (n = 40, developmental stages 53 - 66) was filmed for 10 min both under normal light conditions (see above) and with infrared (IR) illumination (IR Illuminator, TV6700, EcoLine, 850 nm). Because the IR lights also emitted some red light (visible to a human observer), the IR condition most likely was not entirely dark for the animals, but represented a considerably reduced light condition. Half of the animals experienced the normal light condition first, whereas the other half started with IR illumination. Original IR videos lasted 10.5 min and were reduced afterwards to 10 min by removing the first 30 s. The extra 30 s allowed the experimenter to leave the recording room without creating any potentially disturbing light during the 10 min test period.

Convex tank. For the analysis of the swimming behaviour of animals in a convex tank, two of the straight walls of the 19 x 19 cm tank were covered with curvatures. Since the number of swimming episodes along the curved walls was limited, image acquisition was manually started and stopped. Occasionally, animals were gently touched at the tail to stimulate swimming towards the convex curvatures and to redirect the swimming trajectory once the animals got arrested in the concave part of the tank. Images were acquired with the Grasshopper3 camera at a frame rate of 15 fps. Unlike the remaining data (see below), videos were not automatically tracked but visually inspected by the experimenter. A 'trial' was considered as an animal following the wall and swimming past a convex curve, either following the wall or leaving it. Trials were included independent of the body angles of the animal relative to the wall prior to reaching the curve. Trials were excluded if the animal left the wall before reaching the peak of the convex curve. The remaining trials were scored as 'going straight' if the animal departed from the wall at the curve, and as 'following the wall' if the animal continued to follow the wall. The proportion of trials in which the animal swam straight was then calculated for all animals with at least 4 trials.

Tracking of swimming trajectories

Data analysis was carried out by custom-written scripts using Python 3 in the spyder environment (https://github.com/spyder-ide/spyder, version 2.3.8). The main packages included openCV 3 (http://docs.opencv.org/3.0-beta/index.html, version 3.1.0), matplotlib (http://matplotlib.org/, version 1.5.1), numpy (http://www.numpy.org/, version 1.10.4), pandas (http://pandas.pydata.org/, version 0.18.0) and scipy (http://scipy.org/, version 0.17.0). Due to the variety of image file types, image resolution, animal size, illumination conditions and compression quality, the strategy for tracking the animal differed between different sets of experiments. The main difference was that in some cases, background subtraction was carried out before thresholding the image, whereas in other cases images were thresholded directly, either using a simple or a Gaussian threshold.

In contrast, the following steps applied to all cases. The contours of the animals were extracted and the largest contour was taken as the animal. X-Y positions were then calculated relative to the tank geometry. This transformation was achieved by warping the images to the four corners of the tank, which were manually determined. After trajectories were visually inspected, a plot of forward velocity and a video with the animal's position were generated to ensure that the animal was tracked faithfully. Erroneously tracked frames were identified by visual inspection and spuriously high forward velocities, and their X-Y coordinates were interpolated. Such corrections were necessary in 36 video sequences, 22 of which were animals in the standard condition, with maximally 16 frames to interpolate. In some cases, none of the tracking strategies proved successful, leading to an exclusion of 9 animals in the standard condition.

Further data analysis

From the X-Y position in the tank-warped images, parameters such as the distance covered during the swimming and the distance to the nearest wall were calculated. To avoid including jitter as animal movement, the trajectories were simplified with the Ramer-Douglas-Peucker algorithm (using the rdp python package, https://github.com/fhirschmann/rdp). The epsilon parameter, which determines the degree of simplification, was set to 10 in a 900 x 900 pixel video, and was scaled linearly to adjust for changes in the resolution. The simplified trajectory was then used to calculate the total distance the animal covered during swimming. Only animals that covered a distance of at least one side length of the tank were included in the analysis; in the standard condition, this led to the exclusion of four animals. A threshold of 15 mm was chosen to define a 'near wall' area, and the proportion of time that the animal spent near the wall was calculated. While it is desirable to keep the 'near wall' threshold as small as possible, 15 mm was chosen to ensure that the tracked centroid of the large animals was still within that threshold when the animal was near a wall. With 15 mm, the 'near wall' area constituted 29.1% of the 19 x 19 cm tank.

When comparing different tank sizes (7 and 19 cm side length), the animals were compared with a 15 mm 'near wall' threshold – which might indicate the attractiveness of the wall independent of the size of the tank. However, since the 'near wall' area in the 7 x 7 cm tank constitutes 67.3% of the whole tank, the distribution of distances to the wall in both tanks were normalised to the maximum distance, and a threshold was chosen to define the 'near wall' area as intermediate in the proportion between the 29.1% and 67.3% that resulted from the 15 mm threshold. Therefore, 0.28 of the maximal distance from the wall was chosen as a threshold for defining the 'near wall' area independent of the tank's size, yielding a 'near wall' area of 48% in both tanks, which was intermediate between the 'near wall' proportions based on the 15 mm threshold in the two differently sized tanks.

Code and data availability

The python code used to analyse the data and the tracked data can be found on figshare (Hänzi and Straka, 2017a; Hänzi and Straka, 2017b; Hänzi and Straka, 2017c).

Statistics and figures

Parameters of interest were tested for normality using a Shapiro-Wilk test; the appropriate parametric or non-parametric tests were chosen accordingly, using an alpha value of 0.05. The distribution of the proportion of time spent near the wall of all animals in the standard condition was not normally distributed; therefore Spearman rank correlations were used to test relationships to other parameters. Figures were assembled in Adobe Illustrator (Adobe Systems Incorporated, San Jose, USA).

Results

Swimming trajectories of tadpoles and young adult Xenopus

The swimming behaviour of animals in a square tank between pre-metamorphic stage 47 (larvae) and post-metamorphic stage 66 (froglets) was quantified by monitoring the animals' trajectories over a period of 10 minutes in each individual (Fig. 1). Examples of animals at different developmental stages revealed a variety of swimming behaviours with respect to the walls of the tank. Independent of developmental stage, some animals exhibited trajectories that appeared to cover the entire tank (Fig. 2A-C), while others swam preferentially along the walls of the tank (e.g. Fig. 2D,G). To visualise the extent of wall following, the cumulative frequency of distances to the nearest wall over the 10 minutes period of swimming was plotted (see Fig. 1B). This graphical presentation is equivalent to a histogram of distances to the nearest wall that are summed up along the X-axis.

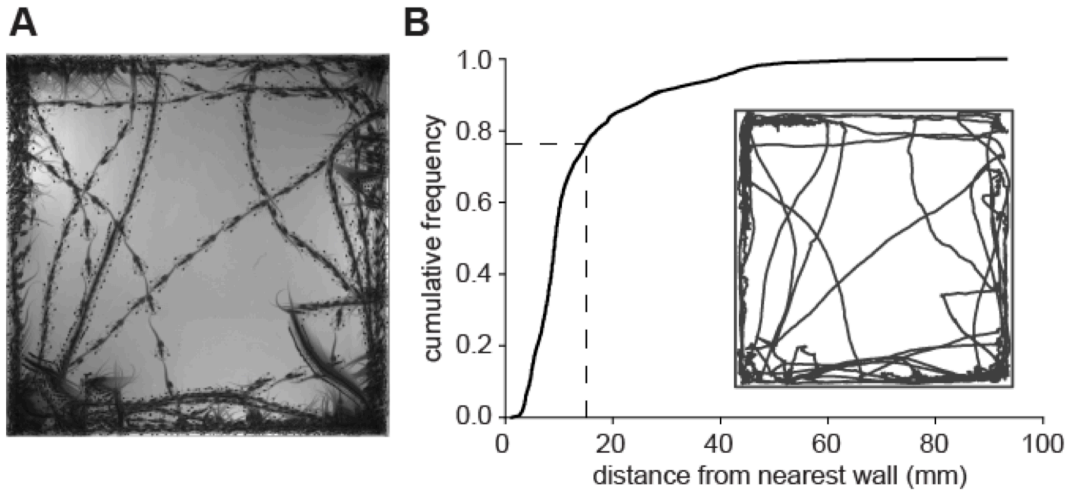

Figure 1. Example swimming trajectory and cumulative frequency distribution of a *Xenopus* tadpole´s distance to the nearest wall. (A) Minimum intensity projection showing the entire trajectory of a stage 54 tadpole (body length 3.6 cm) during swimming in a 19 x 19 cm tank over a 10 min period at a temporal resolution of 3 fps. (B) Cumulative frequency distribution of the animal´s distance to the nearest wall; note that the animal spent over 75% of the time within 15 mm of the nearest wall (dashed lines); the inset shows the tracked trajectory.

The cumulative frequencies of distances to the nearest wall for all animals (n = 79) are shown in Figure 3A.

The proportion of time that the animals spent near the wall (within 15 mm of the wall) was taken as a measure of the strength of wall following. As a group, the 79 animals differed significantly from the proportion that could be expected from the 'near wall' area (29%, Fig. 3B, Wilcoxon signed rank test, $p < 0.0001$). Five animals, however, spent less than 29% of their time near the wall, which is the proportion of the 'near wall' area. Four of these were of developmental stage 48 or below and this tied in well with the impression that the strength of wall following increased with developmental stage (Fig. 3C, Spearman's rank correlation between stage and proportion near the wall, rho = 0.48, $p < 0.0001$, $n = 79$), suggesting that *Xenopus* larvae/froglets become stronger wall followers during ontogeny.

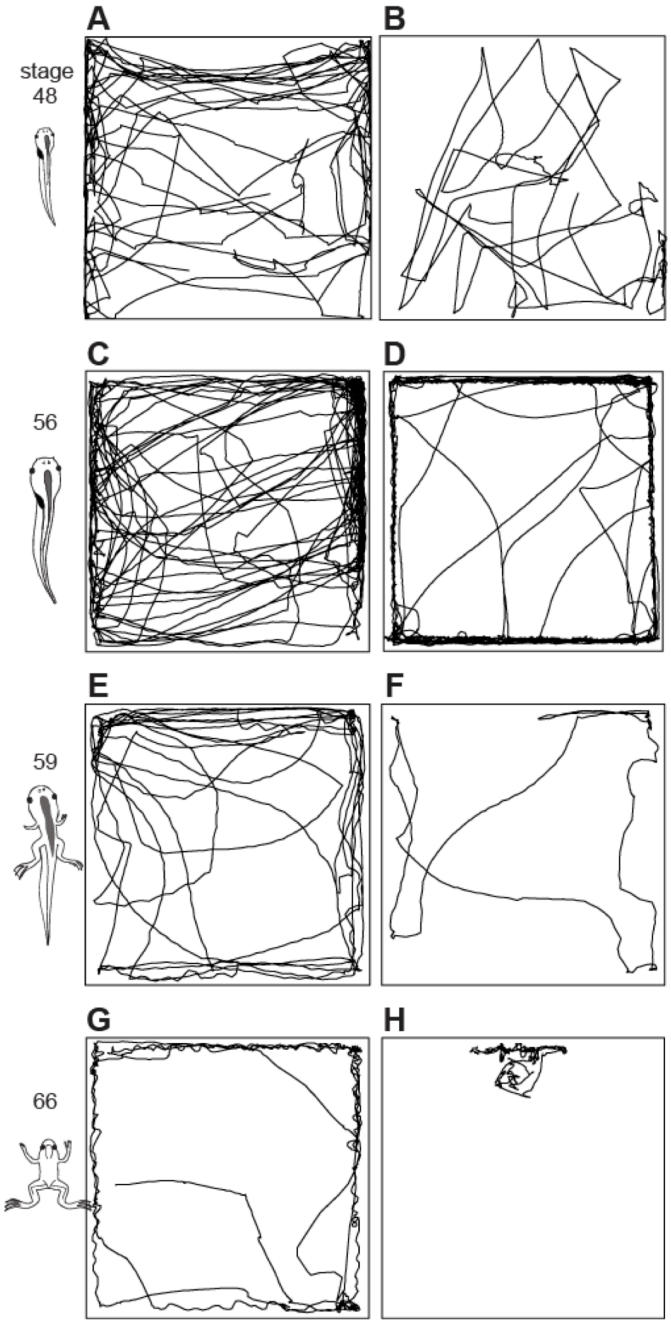

Figure 2. Example swimming trajectories of larval and adult *Xenopus* at different developmental stages. (A-H) Reconstructed trajectories during swimming in a 19 x 19 cm tank over a 10 min period of two animals, respectively, at stage 48 (A,B), stage 56 (C,D), stage 59 (E,F) and of two froglets at stage 66 (G,H). Note the variability of the trajectories of animals at the same developmental stage. The size of the animal schemes on the left (from Hänzi and Straka, 2016) is not related to the spatial dimensions of the trajectories.

To reveal potential changes in wall following behaviour in individual animals over the 10 minute test period, the respective proportions of time spent near the wall were separately calculated for the four quarters of the swimming period (Fig. 3D). Since the proportions of the four quarters were not significantly different from

each other (Fig. 3B, Friedman test, p = 0.29), the individual wall following strategy of a particular animal persisted over the entire test period. Moreover, the total distance covered within the 10 minutes was no confounding factor for wall following, since the rank correlation between the total length of the trajectory and the proportion of time spent near the wall was not significant (Fig. 3E, Spearman's rank correlation, *rho* = 0.03, p = 0.77).

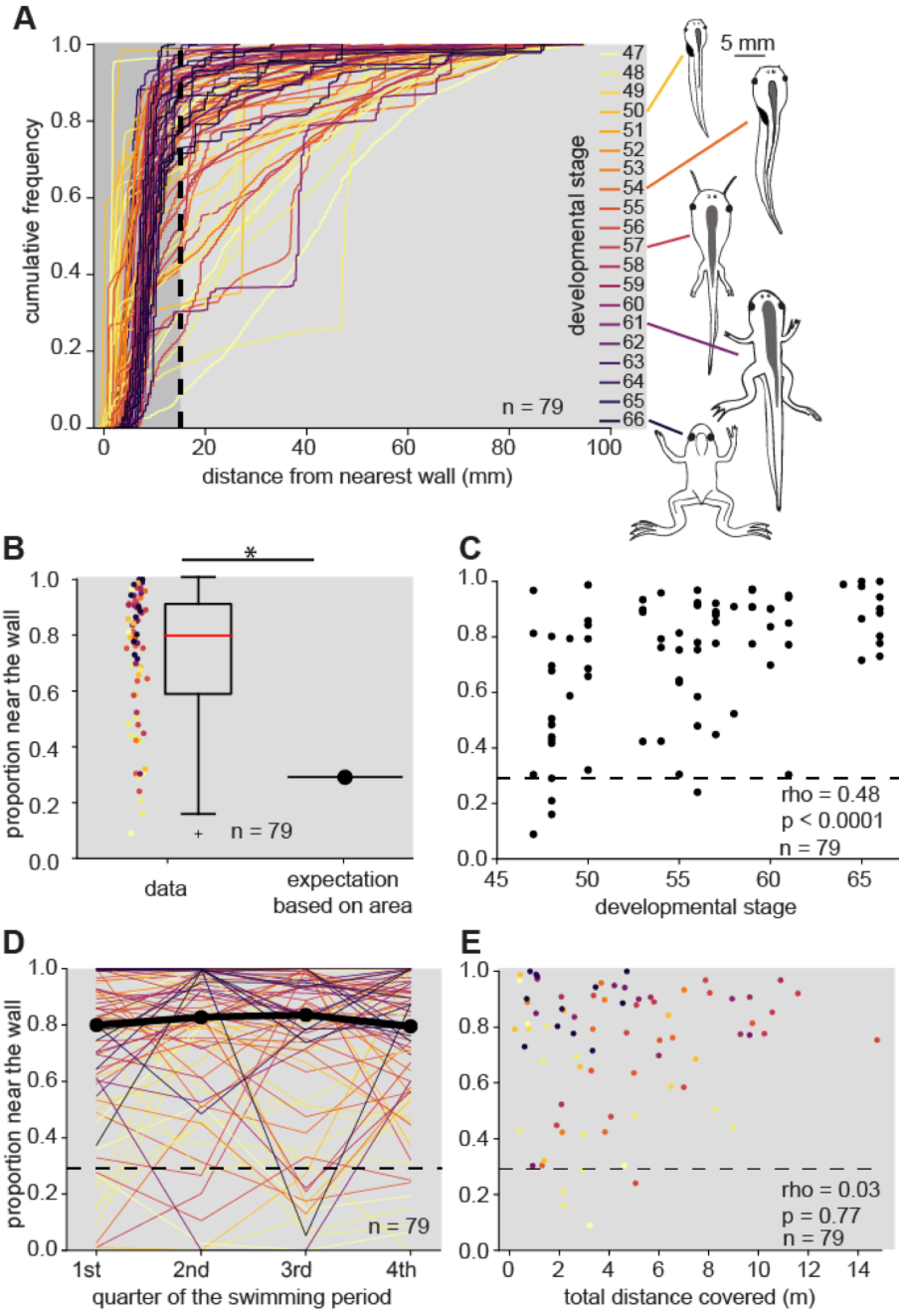

Figure 3. Characterisation of wall following of larval and young adult *Xenopus* during swimming in a square tank. (A) Cumulative frequency distributions of the distance to the nearest wall during swimming of

tadpoles and froglets (n = 79) for 10 min in a 19 x 19 cm tank; traces are colour-coded with respect to developmental stage (colour-code on the right); dashed black line indicates the threshold of the 'near wall' area (15 mm). (B) Proportion of time that the animals spent near the wall from the data shown in A, as colour-coded dots and as a boxplot. The expectation of how much time the animals would spend near the wall based on the 'near wall' area as a proportion of the total area is shown on the right. The animals' proportions were significantly different from this expectation (Wilcoxon signed rank test, $p < 0.0001$, n = 79). (C) Relationship between proportion of time that the animals spent near the wall and the developmental stage of the tested animals (n = 79); note the significant Spearman's rank correlation between stage and one-sample KS statistics (n = 79, rho = 0.48, $p < 0.0001$), indicating that older animals are stronger wall followers. (D) Separate proportion of the time that the animal spent near the wall for each quarter of the 10 min swimming episode shown in A (n = 79, colour-coded for developmental stage). The median across all animals is shown as a thick black line. These proportions did not change significantly across the four quarters of the 10 min swimming period (Friedman test, p = 0.29). (E) Relationship between the proportion the animals spent near the wall and the total distance covered by an animal over the 10 min swimming period (colour-coded for developmental stage). The absence of significance (Spearman's rank correlation, rho = 0.03, p = 0.77) indicates that total covered distance is not a confounding factor for the degree of wall following as measured by the proportion of the time spent near the wall. The dashed line in C-E indicates the 'near wall' area as a proportion of the total tank area. Schemes of *Xenopus* in A from (Hänzi and Straka, 2016).

Role of tentacles in wall following behaviour

During larval development between stage 51 and 60, *Xenopus laevis* tadpoles transiently posses a mobile pair of rod-like appendages that protrude from the corners of their mouths (Nieuwkoop and Faber, 1956). These appendages might be necessary or at least advantageous for wall following, given the presence of Merkel cells, potentially assigning a tactile function to these tentacles (Ovalle, 1979; Ovalle et al., 1998). However, contrasting with normal development, a number of animals from our breeding facility failed to naturally develop noticeable tentacles. This allowed to directly test the influence of tentacles on the degree of wall following. Accordingly, the swimming behaviour of a population of tadpoles at developmental stages 54 - 60 without appendages (n = 11) was compared with that of an age-matched group of tadpoles (n = 13) that possessed tentacles with a length of at least 3 mm.

Statistical analysis of the swimming behaviour as reported above indicated that both populations of animals had a similar propensity for wall following (blue and red traces in Fig. 4A). This is demonstrated by the overlapping distributions of the cumulative frequencies of distances to the nearest wall in animals with and without tentacles (blue and red traces in Fig. 4A). The proportions of time that these animals spent near the wall were not significantly different between animals with and without tentacles (Fig. 4B, Mann-Whitney-U test, p = 0.09). If anything, animals without tentacles were located closer to the wall than animals with tentacles (see blue and red traces in inset in Fig. 4A). This likely derives from the fact that the presence of tentacles creates an additional distance of the tadpole with respect to the wall that is not present in animals without tentacles. Tentacles are therefore no prerequisite for wall following. This, however, does not exclude that tentacles are used as tactile probes; rather it shows that despite the absence of tentacles, tadpoles follow the walls of a tank and potentially use facial skin areas as tactile probes.

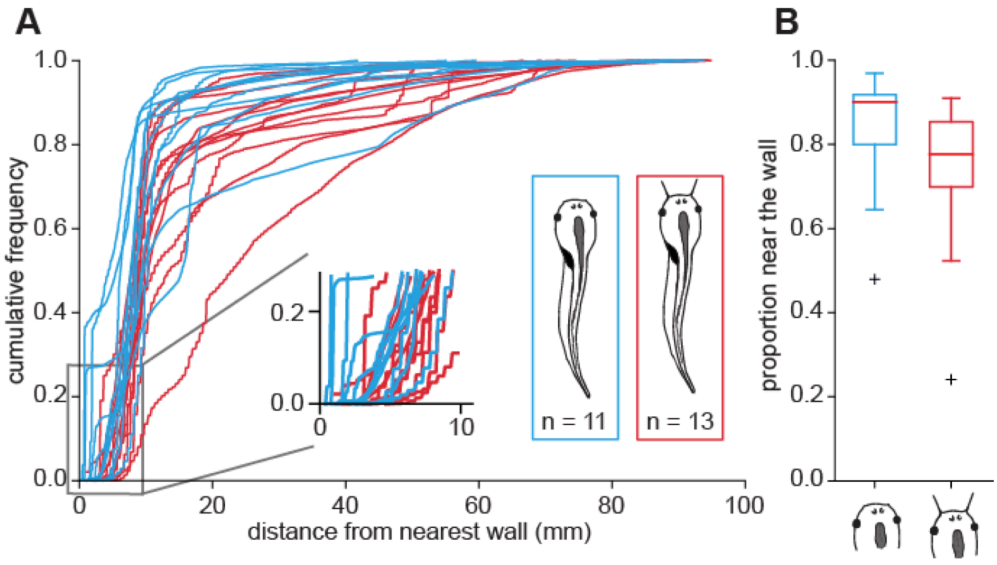

Figure 4. Influence of tentacles on wall following during swimming in *Xenopus* larvae. (A) Cumulative frequency distributions of the distance to the nearest wall of animals with tentacles (red, n = 13) and of animals without tentacles (blue, n = 11) between developmental stages 54 – 60; the inset is a higher magnification of the initial part of the cumulative frequency distribution and shows that tadpoles without tentacles (blue) align closer with the wall compared to tadpoles with tentacles (red). (B) Proportion of the time that the animals with and without tentacles spent near the wall; the two groups were not significantly different (Mann-Whitney U test, p = 0.09).

Wall following under different luminance conditions

The wall following of *Xenopus* larvae/froglets analysed above was further examined during swimming under different illumination conditions, which could have facilitated or impaired wall detection. A potential influence of the visual system was therefore evaluated in a separate set of experiments where the swimming of stage 50 - 65 tadpoles/froglets (n = 10) was compared in a tank in which the four walls were covered on the outside by a white or a black background (Fig. 5A,B). Analysis of the swimming behaviour indicated that the propensity for wall following was not related to the background (Fig. 5B) based on the proportions of time that each animal spent near the wall in the two conditions (paired t-test, p = 0.59). This suggests that the visual system exerts no apparent influence on the tendency of *Xenopus* for wall following. This conclusion was confirmed by another set of experiments in which the swimming behaviour of tadpoles/froglets (n = 30, stage 53-66) was tested under both white light (cold light source) and infrared illumination (850 nm, Fig. 5C,D). Analysis of the proportion of time spent near the wall revealed no significant difference between the two conditions (Fig. 5D, paired Wilcoxon signed-rank test, p = 0.47), indicating that the reduced light condition during infrared illumination had no effect on wall following.

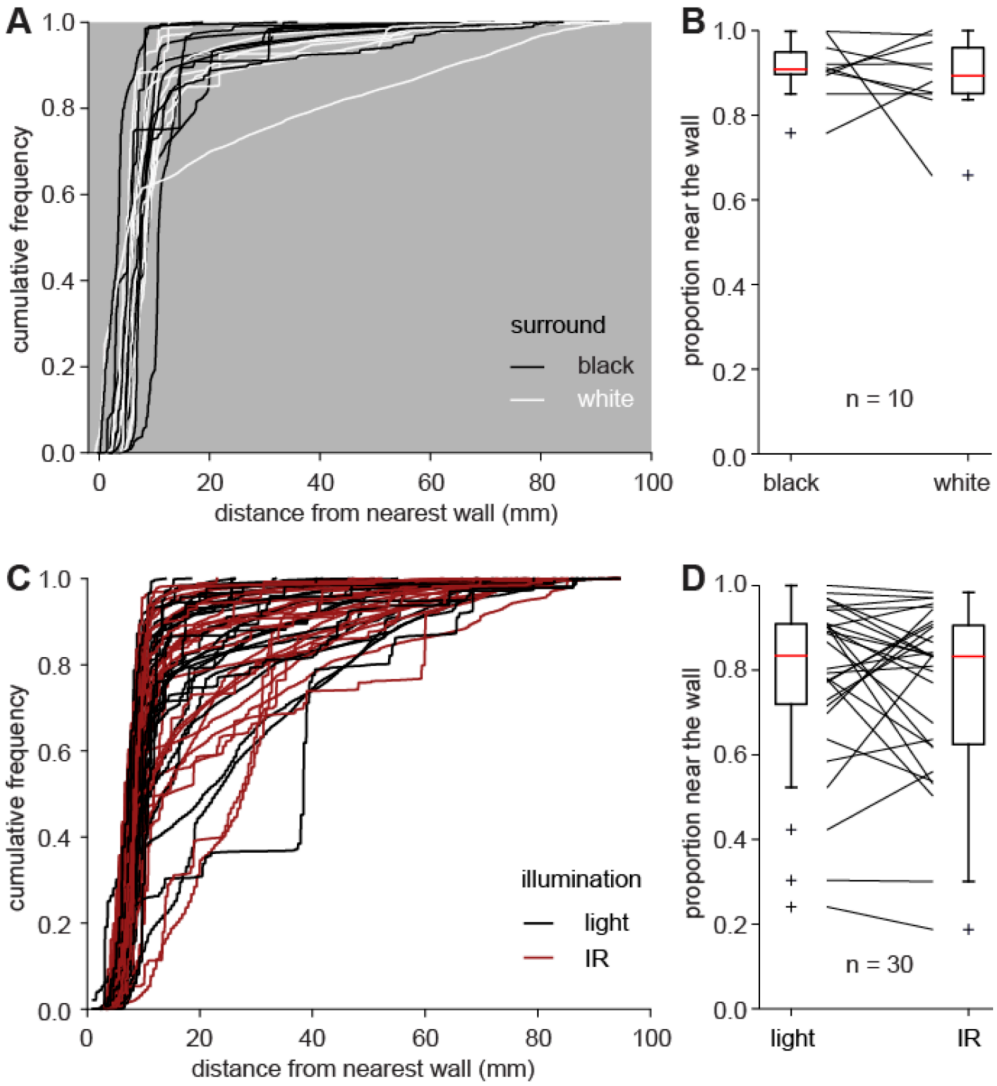

Figure 5. Influence of illumination conditions on wall following during swimming in *Xenopus* larvae. (A) Cumulative frequency distributions of the distance to the nearest wall during swimming of stage 53 – 65 tadpoles/froglets (n = 10) over a 10 min period in a 19 x 19 cm tank surrounded by black (black traces) or white paper (white traces). (B) Proportion of time that the animals spent near the wall (within 15 mm) for swimming in the tank surrounded by black (left) or white (right) paper. The proportions in these two conditions were not significantly different (paired t-test, p = 0.59). (C) Cumulative frequency distributions of the distance to the nearest wall during swimming of stage 50 – 65 tadpoles/froglets (n = 30) over a 10 min period in a 19 x 19 cm tank illuminated either with cold light (light, black traces) or infrared light (IR, red traces). (D) Proportion of the time that the animals spent near the wall (within 15 mm) for swimming in the tank with cold light (left) or IR light (right). The proportions in these two illumination conditions were not significantly different (paired Wilcoxon signed-rank test, p = 0.47).

Influence of tank size on wall following

Wall following might be influenced by the size of the environment. To test whether the wall is equally attractive independent of the size of the tank, animals of developmental stages 47 – 50 (n = 9) were tested both in a 19 x 19 cm and in a 7 x 7 cm tank. The cumulative frequency distributions of distances to the nearest wall

suggest that the animals spend more time near the wall in the smaller tank (Fig. 6A). This is confirmed by comparing the proportion of time that the animals spent near the wall (within 15 mm of the wall) in the two tanks: the proportions in the small tank are significantly larger (Fig. 6B, paired Wilcoxon signed rank test, p = 0.0078). This suggests that the wall is more attractive in the smaller tank. However, the 'near wall' area (within 15 mm of the wall) is also relatively larger in the smaller tank (67.3% of the total area in the 7 x 7 cm tank vs. 29.1% of the total area in the 19 x 19 cm tank). To compare wall following on the same scale, the distances to the wall were normalised to their maximum, and a threshold was chosen that resulted in an intermediate 'near wall' area (threshold of 28% of the maximal distance to the wall, resulting in a 'near wall' area of 48% of the total tank area; Fig. 6C). The proportion of time spent in these area-normalised 'near wall' areas was again significantly larger in the smaller tank (Fig. 6D, paired Wilcoxon signed rank test, p = 0.0078). The animals are therefore stronger wall followers in the smaller tank also when taking into account the differences in area.

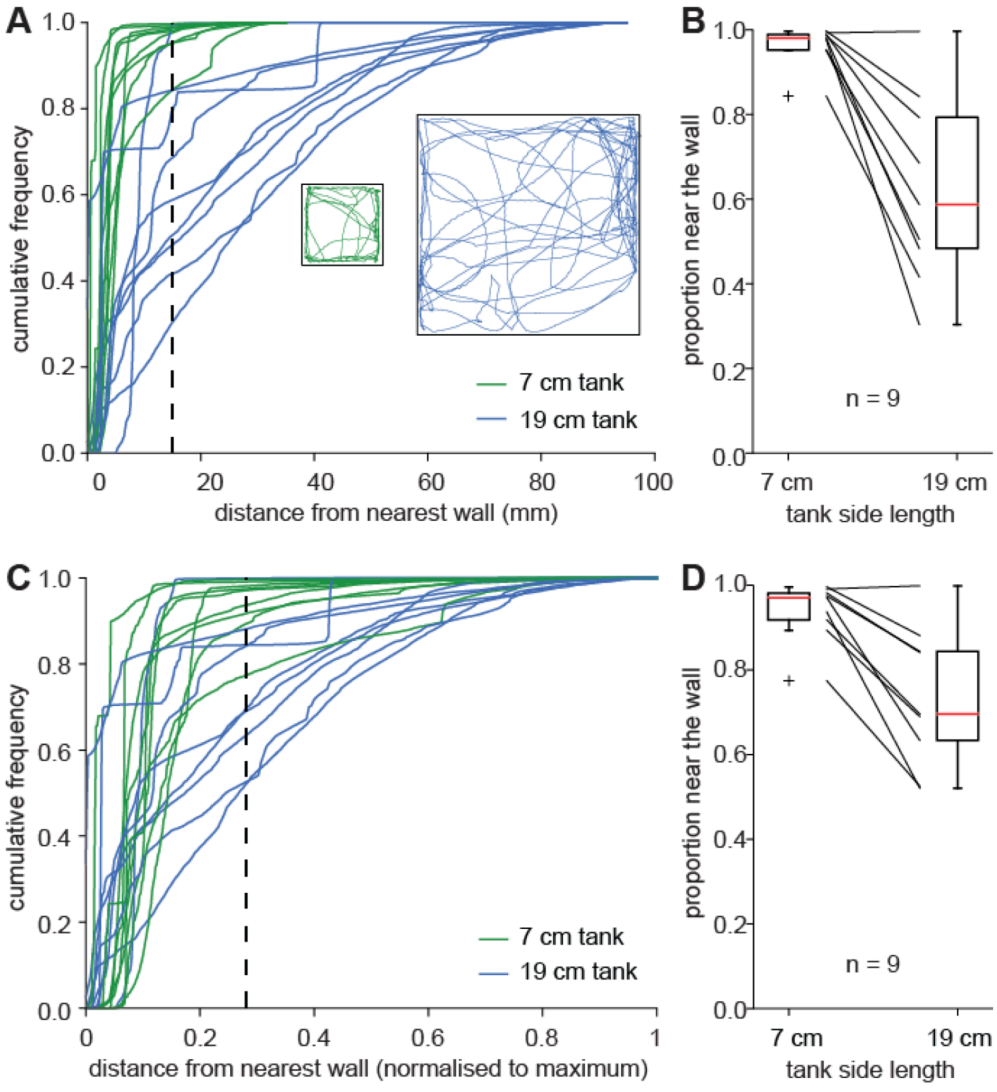

Figure 6. Influence of tank size on wall following. (A) Cumulative frequency distributions of the distance to

the nearest wall during swimming of stage 47 – 50 tadpoles (n = 9) over a 10 min period in a 7 x 7 cm tank (green) and in a 19 x 19 cm tank (blue). The 'near wall' threshold (15 mm) is shown as a black dashed line. The trajectories of a stage 50 tadpole (3.2 cm body length) are shown as insets (B) Proportion of time that the animals in A spend near the wall (within 15 mm); the two groups were significantly different (paired Wilcoxon signed rank test, p = 0.0078, n = 9). (C) Same data as in A but normalised to the maximal distance to the wall. The black dashed line indicates the threshold (28% of the maximal distance to the wall) that yields a 'near wall' area intermediate to what 15 mm yields in the 7 and 19 cm tank (see Methods). (D) Proportion of time the animals spend near the wall (within 28% of the maximal distance) in the tanks with a side length of 7 and 19 cm; the two groups were significantly different (paired Wilcoxon signed rank test, p = 0.0078, n = 9).

Wall following is passive

Wall following might be either active such as in blind cavefish (Patton et al., 2010) or passive (distinction according to Creed and Miller, 1990). To distinguish between the two possibilities for wall following in larval and adult *Xenopus*, the swimming behaviour was tested in a specifically designed tank (Fig. 7A,B). The use of a tank in which two of the four walls had convex curvatures allowed testing if tadpoles seek wall touch during swimming actively or follow concave walls passively (red and blue arrows in Fig. 7A). The proportion of trials when animals swam straight after encountering a convex curve (Fig. 7B) was evaluated from visual inspection by the experimenter. The majority of tested tadpoles swam straight in all trials (Fig. 7B) more or less independent of their developmental stage (Fig. 7C,D, n = 22), leading to the conclusion that wall following in *Xenopus* is passive.

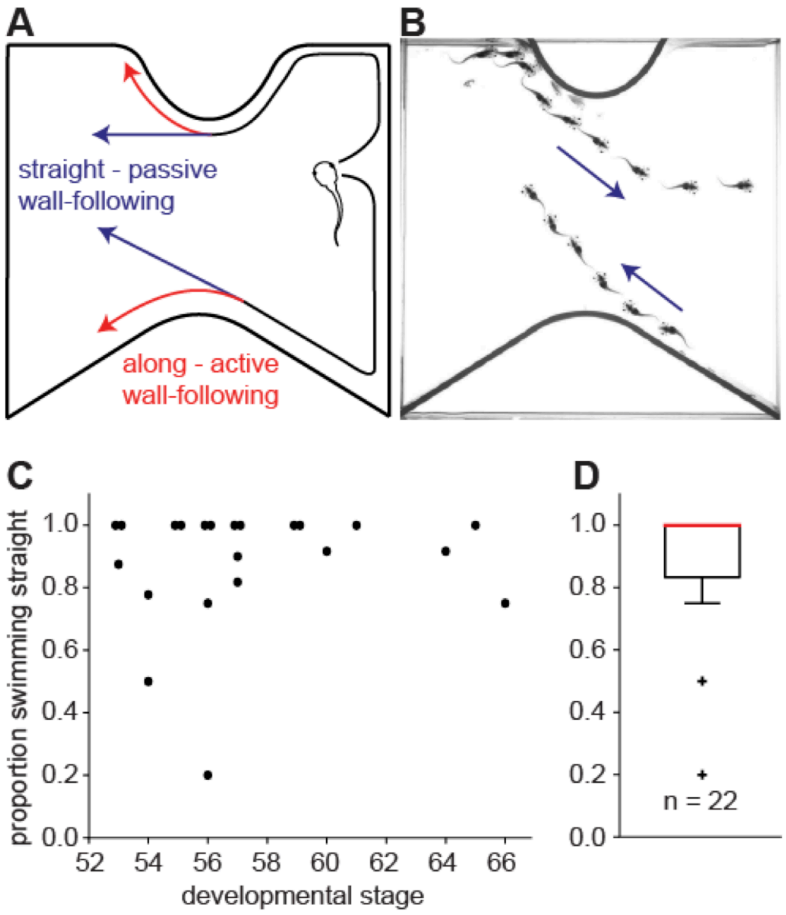

Figure 7. Wall following is passive. (A) Tank (19 x 19 cm) with two convex walls to distinguish if wall following is active (red arrows) or passive (blue arrows). (B) Minimum intensity overlay (at a frame rate of 3 fps) of two swimming trajectories along the curved walls of a stage 55 tadpole; blue arrows indicate the animal's direction of swimming. (C) Proportion of trials with straight swimming and departure from the wall in animals at different developmental stages. (D) Boxplot of the proportion of straight swimming across all animals (n = 22). In C and D, only animals with at least 4 trials were included.

Discussion

Xenopus laevis – from small tadpoles to froglets – tend to follow the wall when swimming in a square tank. The strength of wall following increases with progressive development and smaller tank size and is not confounded by the total distance that an animal covers. The transient presence of mechanosensory tentacles at mid-larval stages does not lead to stronger wall following compared to animals that naturally do not develop these appendages. Also, vision is unlikely a main driver of wall following, as surrounding the tank by black or white paper or changing the light to infrared illumination does not change the strength of wall following. Wall following is passive as indicated by straight swimming in a tank with convex curvatures. This indicates that wall following in *Xenopus* is likely imposed by the concave environment. Wall following being passive might also explain why it persists across metamorphosis and is present in both tadpoles and froglets, independent of their very different locomotor styles.

Classification and different types of wall following

Wall following in concave environments has been described for a wide variety of animals: from crustaceans such as crayfish (Basil and Sandeman, 1999) to insects such as *Drosophila* (Besson and Martin, 2005; Martin, 2004), or cockroaches (Camhi and Johnson, 1999; Jeanson et al., 2003; Okada and Toh, 2000), to fishes such as zebrafish (Anichtchik et al., 2004; Colwill and Creton, 2011), goldfish (Kato et al., 1996), salmon (Clements et al., 2002) or blind cavefish (Patton et al., 2010; Teyke, 1985; Teyke, 1989), to several rodent species including voles, rats and mice (Eilam, 2004; Perrot-Sinal et al., 1999; Simon et al., 1994; Treit and Fundytus, 1988; Webster et al., 1979; Wilson et al., 1976). In many cases, these examples of wall following behaviours have been described in the context of thigmotaxis and centrophobism, and in relation to the level of anxiety. Thigmotaxis is a term that describes the motion of an organism relative to a touch stimulus; it is often used as shorthand for positive thigmotaxis, which means that animals actively seek out touch stimulation as they move. Centrophobism, on the other hand, is a tendency of animals to avoid open spaces, for instance the centre of an open test field for mice or rats (Martínez et al., 2002). Some authors use the term centrophobism when the avoidance of open spaces is related to vision (Cardenas et al., 2001). For instance common spiny mice move much more often into an open space in the dark than in the light (Eilam, 2004), though some authors use the term centrophobism without necessarily implying a visual mechanism. Thus, centrophobism and thigmotaxis are two potential but not mutually exclusive mechanisms that can lead to the avoidance of open spaces and the following of environmental boundaries. Wall following is therefore a neutral term to describe the tendency of an animal to follow vertical walls in its environment without a reference to the underlying mechanism. An environment with convex borders allows distinguishing between passive and active wall following (Creed and Miller, 1990). Animals perform active wall following when voluntarily seeking out the proximity to a wall and turn in order to remain near the wall. Passive wall following occurs when animals leave the wall at a convex curve but follow the walls in a concave environment. When wall following is active, thigmotaxis, centrophobism or a combination of the two can be the underlying mechanism.

Potential uses of wall following

Thigmotaxis has been described both as a defensive strategy (Grossen and Kelley, 1972) as well as a spatial exploration strategy (Kallai et al., 2007). Animals might be safer near a vertical wall compared to the open; for instance it has been suggested that avian predation on rats likely is lower near a wall than in the open (Grossen and Kelley, 1972). Mice increase thigmotaxis in the presence of a potential predator (Bonsignore et al., 2008). Other rodents such as the common spiny mouse only venture in the centre of an open field if there are objects that might serve as shelter, or if it is dark (Eilam, 2004). Moreover, thigmotaxis has been related to anxiety, and is commonly used as a simple behavioural readout of anxiety levels in mice and rats (Prut and Belzung, 2003; Simon et al., 1994; Treit and Fundytus, 1988). Some authors argue that fear of open spaces is not only driven by touch but also by vision (Martínez et al., 2002), which suggests that wall hugging and avoidance of open spaces is a combination of thigmotaxis and centrophobism. Independent of the underlying mechanisms, the use as a defensive strategy is clear. Moreover, wall following can also serve as a useful spatial exploration strategy. Especially under conditions when long-range sensing such as vision is not available, exploration of the environment based on touch along its borders can provide the basis for the formation of a cognitive map (Kallai et al., 2007; Yaski et al., 2009) and serve as a reference frame for later exploration (Kallai et al., 2005). However, this is only useful as an initial strategy; if it is used excessively it can even prevent further spatial learning (Kallai et al., 2007). Such initial wall following as a means for spatial learning has been observed in various species such as crayfish (Basil and Sandeman, 1999), blind cavefish (Teyke, 1989), and blind mole rats (Avni et al., 2008).

Persistence of wall following with development in Xenopus

In this study we examined a range of developmental stages of *Xenopus* – from small to large tadpoles immediately prior to metamorphosis as well as froglets after metamorphosis has been completed. Wall following in a square tank was present at all developmental stages; the strength of wall following was weakest, however, in the smallest tadpoles, stronger, with considerable variations in larger tadpoles and consistently strong in froglets. This persistence suggests that wall following is not a behavioural strategy only employed by tadpoles or frogs, and is not linked to a particular locomotor style such as undulatory tail-based propulsion or leg-based swimming. Moreover, wall following in a convex tank was passive in all animals tested (see below). The weaker wall following in young larvae is noticeable and might be related to the somewhat different swimming style of these animals (see Fig. 3A in Hänzi and Straka, 2017), where the rotation axis of the left-right head undulations oscillates between positions outside the animal; this is at variance with the situation in larger tadpoles where the head oscillations during swimming occur around a single central axis (Lambert et al., 2009). This difference in swimming style might facilitate turns away from a vertical wall in young larvae and explain the weaker wall following.

At intermediate developmental stages examined in this study (stage 51-60 according to Nieuwkoop and Faber, (1956)), tadpoles normally possess a pair of mobile appendages protruding from the corners of their

mouths, which are retracted during undulatory swimming (Hänzi et al., 2015). These tentacles – like other skin areas – possess mechanoreceptive Merkel cells (Nurse et al., 1983; Ovalle, 1979; Ovalle et al., 1998), and therefore the tentacles likely serve a tactile function when the animal is stationary or cruising slowly with tentacles extended forward. We hypothesised that these tentacles might be used to explore the environment in a way that is similar to rodents' whiskers but simpler because the structure is not as specialised. However, younger larvae and older animals at metamorphic climax (>stage 61) that do not possess any tentacles were overall similar in their wall following tendencies, as were animals that for unknown reasons did not develop tentacles (Fig. 4). While this does not exclude that – when present – tentacles are used for tactile exploration, it shows at least that tentacles are not necessary for wall following, and if tactile exploration is needed, tadpoles might also use their facial skin.

Effects of vision

As mentioned above, some rodents leave the walls and venture much more into open space in darkness than in light; this is true not only for the common spiny mouse (Eilam, 2004) but also for rats (Nasello et al., 1998), some types of gerbil (Zadicario et al., 2005) and wild-caught prairie deer mice (Brillahart and Kaufman, 1991). Some rodents also adjust their foraging behaviour in laboratory or natural conditions such that they venture more into the open in the dark (Diaz, 1992; Price et al., 1984; Vasquez, 1996), and some authors also assign a role of vision in the avoidance of open spaces by rats (Cardenas et al., 2001; Martínez et al., 2002). However, tadpoles and froglets of *Xenopus laevis* did not show stronger wall following in light than under infrared illumination. While the IR lamps used here did not produce pure infrared light, IR illumination nevertheless is a condition with considerably reduced light and influence of vision. Centrophobism or visually driven fear of open spaces is therefore very unlikely to be the driving force behind wall following in *Xenopus*. The wall following strategy might rather be a side effect of locomotion in the mostly murky aquatic environment of the natural habitat of *Xenopus* (Nieuwkoop and Faber, 1956) independent of the developmental stage.

Effects of the size of the environment

A range of different arena sizes have been used in rodent open field tests (Walsh and Cummins, 1976), and the geometry of the environment has shown to influence path shapes of rats not only at the perimeter but also at the centre of an environment (Yaski et al., 2011). A wall can exert both a guiding and attracting influence on mouse behaviour from quite some distance (Horev et al., 2007). Two studies explicitly examined the proportion of time that social voles spend near the wall in arenas of different sizes (Eilam, 2003; Eilam et al., 2003). These animals are very active, and spend more time near the wall in larger arenas – possibly because the larger open space is perceived as more dangerous than a smaller, more enclosed open space. This contrasts with the behaviour of *Xenopus* described here, which are stronger wall followers in smaller tanks. It therefore seems likely that wall following in *Xenopus* is imposed by the constraints of the environment, whereas wall following in social voles serves as a defensive strategy. Moreover, thigmotaxis is unlikely to be the main mechanism behind wall following in either of the two cases, since different tank/arena sizes would have no impact on wall

following if thigmotaxis was the underlying cause (Eilam et al., 2003) – with thigmotaxis as the main mechanism, the walls would be equally attractive independent of the arena size.

Passive versus active wall following

The studies ascribing protection or exploration as the function of wall following have used the terms thigmotaxis or centrophobism for a reason: wall following can only be protective or exploratory if it is active. Passive wall following such as observed in this study in *Xenopus laevis* is rather unlikely to serve these purposes. To the best of our knowledge, no other study described passive wall following so far. Potential reasons include that only few studies use convex tanks, and that passive wall following might be considered a negative finding and not be reported. The few following studies did use convex enclosures to discriminate active from passive wall following: In blind cavefish, for instance, wall following is clearly active (Patton et al., 2010; Sharma et al., 2009). These animals are blind, live in dark caves and use their lateral line system as a near range sense to obtain information about their environment. In a convex tank they actively follow the wall because they would not be able to orient otherwise. Adult fruit flies, on the other hand, leave the wall in more than 50% of the trials; their preference for walls in circular arenas seems to derive from a preference for the boundaries of the environment rather than from thigmotaxis or centrophobism (Soibam et al., 2012). In contrast to fruit flies, cockroaches have antennae that can be longer than their body (Camhi and Johnson, 1999). These animals use these mechanoreceptive sensors to gain information about their nearby environment. Cockroaches thus have been described as thigmotactic in concave environments (Camhi and Johnson, 1999; Jeanson et al., 2003) and show positive thigmotaxis towards objects that are touched by the antennae (Okada and Toh, 2000). When running along a wall these animals constantly touch the wall with one of their antennae (Camhi and Johnson, 1999). However, when arriving at a convex curve, they leave the curve in about 50% of the trials (Creed and Miller, 1990).

Active wall following in blind cavefish certainly serves as a spatial exploration and spatial learning strategy (Teyke, 1989), and to a certain extent this might also be true for cockroaches or fruit flies. In contrast, wall following in *Xenopus laevis* is passive and therefore unlikely to serve as a specific protective or exploratory strategy or a behaviour that is related to anxiety. A number of factors potentially influencing wall following such as changes in illumination or the presence of tentacles were shown to play no major role for wall following in *Xenopus*. Instead, passive wall following in these animals might be due to the particularity of the rather unnatural and concave test environment. This thus suggests that spatially more complex and natural environments likely would yield richer behaviours (see also Benjamini et al., 2010; Cheng, 2005).

List of abbreviations: IR: infrared.

Acknowledgements

The authors thank all members of the Straka lab for feedback and discussion.

Competing interests

No competing interests are declared.

Author contributions

Investigation, Software, Visualization: S.H.; Supervision, Project administration, Funding acquisition: H.S.; Conceptualization, Writing: S.H. and H.S.

Funding

This study was funded by the German Science Foundation (STR 478/3-1) and the German Federal Ministry of Education and Research under the grant number 01 EO 0901.

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3 Head movements during swimming

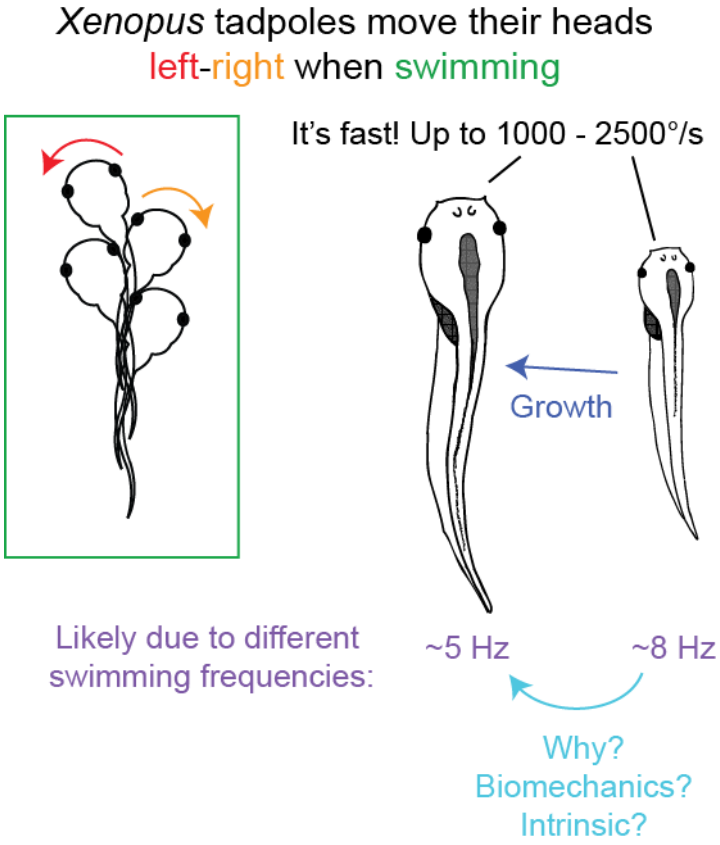

Figure 5. Graphical abstract for the study on head movement kinematics. Tadpole schemes on the right from Hänzi and Straka (2016b).

3.1 Citation

Hänzi, S., and Straka, H. (2017b). Developmental changes in head movement kinematics during swimming in *Xenopus laevis* tadpoles. J. Exp. Biol. *220*, 227–236.

3.2 Contributions

Investigation, Software, Visualization: S.H.; Supervision, Project administration, Funding acquisition: H.S.; Conceptualization, Writing: S.H. and H.S.

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RESEARCH ARTICLE

Developmental changes in head movement kinematics during swimming in *Xenopus laevis* tadpoles

Sara Hänzi[1,2,*] and Hans Straka[1]

ABSTRACT

During the post-embryonic developmental growth of animals, a number of physiological parameters such as locomotor performance, dynamics and behavioural repertoire are adjusted to match the requirements determined by changes in body size, proportions and shape. Moreover, changes in movement parameters also cause changes in the dynamics of self-generated sensory stimuli, to which motion-detecting sensory systems have to adapt. Here, we examined head movements and swimming kinematics of *Xenopus laevis* tadpoles with a body length of 10–45 mm (developmental stage 46–54) and compared these parameters with fictive swimming, recorded as ventral root activity in semi-intact *in vitro* preparations. Head movement kinematics was extracted from high-speed video recordings of freely swimming tadpoles. Analysis of these locomotor episodes indicated that the swimming frequency decreased with development, along with the angular velocity and acceleration of the head, which represent self-generated vestibular stimuli. In contrast, neither head oscillation amplitude nor forward velocity changed with development despite the ∼3-fold increase in body size. The comparison between free and fictive locomotor dynamics revealed very similar swimming frequencies for similarly sized animals, including a comparable developmental decrease of the swimming frequency. Body morphology and the motor output rhythm of the spinal central pattern generator therefore develop concurrently. This study thus describes development-specific naturalistic head motion profiles, which form the basis for more natural stimuli in future studies probing the vestibular system.

KEY WORDS: Locomotion, Tadpole, Body morphology, Central pattern generator, Vestibular system

INTRODUCTION

During development, when animals grow in size with species-specific modifications of body size, form and proportion, the dynamics of the locomotor mechanisms and the sensory feedback have to concurrently adapt to maintain or even improve motor performance. Specific modifications of propulsive structural elements and motion detection systems are required to ensure both optimal motor output and maintenance of an adequate sensitivity and working range of the sensory organs, as self-motion activates responses of various sensory modalities such as the visual and vestibular systems (Carriot et al., 2014; Wark et al., 2007). Thus, quantification of locomotor performance allows the extraction of species-specific kinematic parameters of the propulsive mechanisms as well as inference of the stimulus statistics of self-generated sensory inputs. Moreover, comparing locomotor characteristics during development – in particular, in fish and amphibians, which develop through a series of fast-growing larval stages with changes in body proportions and form – allows alterations in body size and sensory capacity to be related to concurrent changes in locomotor dynamics.

Many studies have added to the current knowledge of the development of the motor program underlying free swimming in *Xenopus laevis* tadpoles (see Roberts et al., 2010), zebrafish (Brustein et al., 2003; Saint-Amant and Drapeau, 1998) and angelfish (Yoshida et al., 1996), on both a behavioural and a neural circuit level. However, little is known about how the kinematic parameters of swimming change during larval development, when the relative body proportions change and the size of the animals and their appendages increase considerably. While a previous anecdotal report described some aspects of locomotion in a very small number of older *Xenopus laevis* tadpoles at an unspecified developmental stage (Hoff and Wassersug, 1986), a systematic evaluation of head/body movements and quantification of alterations in swimming kinematics during larval development is lacking. A major advantage of using *X. laevis* for such an analysis is the solid knowledge about the embryonic and early larval development of the spinal motor circuitry (Harland and Grainger, 2011; Roberts et al., 2010; Wallingford et al., 2010). In addition, the ability to perform *in vitro* experiments on isolated semi-intact preparations (Straka and Simmers, 2012; Straka et al., 2016) allowed the consequences of locomotor activity on the processing of head/body motion-related sensory signals to be studied (Chagnaud et al., 2015; Lambert et al., 2012a). A description of developmental changes in locomotor dynamics during larval life will facilitate the understanding of how swimming kinematics and the capacity of vestibular sense organs for motion detection might influence each other.

Here, we used *X. laevis* tadpoles with a length of 10–45 mm (developmental stages 46–56 according to Nieuwkoop and Faber, 1956) to reveal how swimming kinematics change during the period when the animals experience their largest growth in body size. The movements of the larvae in the horizontal plane during episodes of free swimming were captured with a video camera from above to quantify locomotor parameters such as swimming frequency and forward velocity, as well as angular velocity and acceleration, from the tightly coupled head/tail movements that are relevant as stimuli for the activation of vestibular organs (Chagnaud et al., 2012). The obtained natural stimulus statistics of self-generated vestibular signals allowed estimation of the size-related impact of self-motion on the activation of responses in hair cells of the semicircular canal organs. Comparison of the characteristics of locomotor performance during free and fictive swimming – the latter obtained from electrophysiological recordings in stationary *in vitro* preparations – addressed the question of whether any potential changes in locomotor parameters during development required online sensory feedback

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Received 19 July 2016; Accepted 23 October 2016

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(in which case the change would be a short-term adaptation according to the terminology of Pearson, 2000), or whether these changes persisted in the absence of immediate sensory feedback, in which case they would be long-term adaptations.

MATERIALS AND METHODS

Animals

Experiments were performed on tadpoles of the African clawed toad, *Xenopus laevis* Daudin 1802, of either sex at developmental stages 46–56 (Nieuwkoop and Faber, 1956) with a size of 10–45 mm. Animals at different developmental stages were identified according to stage-specific morphological features in freely moving animals in a Petri dish under a dissection microscope. Tadpoles were obtained from the in-house breeding facility at the Biocentre of the Ludwig-Maximilians-University Munich, where all animals were kept in aerated tanks at 17°C on a 12 h:12 h light:dark cycle. All behavioural observations as well as electrophysiological experiments on isolated, semi-intact *in vitro* preparations complied with the 'Principles of animal care', publication no. 86-23, revised 1985, of the National Institutes of Health. Permission for experiments was granted by the Regierung von Oberbayern (55.2-1-54-2532.3-59-12).

Video recordings of locomotor activity

The locomotor performance of tadpoles (N=25 animals) was evaluated by video recordings of the swimming activity in a 7×7 cm tank with a water depth of 1–1.2 cm. The water was maintained at a constant temperature of 17°C by placing the tank into a larger (20×20 cm) container, filled with approximately 1 litre of water, that allowed regulation of the temperature. Videos were acquired with a Point Grey Grasshopper3 camera (GS3-U3-23S6M-C, Point Grey, Richmond, Canada) using Fly Capture software (version 2.8.3.1). The spatial resolution was 960×960 pixels, and the temporal resolution was ∼200 frames s^{-1}; the images were saved as series of separate tiff files. The time stamp of each frame was read out individually from the camera's internal cycle running at 8 kHz. Animals in the tank were filmed from above with lighting provided from below (ZLED CLS6000, Zett Optics GmbH, Braunschweig, Germany). Recordings were started and terminated manually to maximise recording episodes of swimming events when the animal was not touching one of the four vertical walls. While on some occasions the animals spontaneously swam through the centre of the tank, most of the time the tadpoles followed a trajectory along the vertical walls. In order to increase the number of unimpaired swimming episodes through the centre of the tank, animals were gently touched to redirect the swimming trajectory.

Data analysis

The tiff files from the videos were opened in Fiji (http://fiji.sc/Fiji) and visually inspected for wall touch by the tadpoles. Episodes without wall touch were further analysed by thresholding the images such that the eyes formed distinct black shapes. These objects were tracked using the MTrack2 plugin (http://valelab.ucsf.edu/~nstuurman/ijplugins/MTrack2.html; Fig. 1A) and the tracking results were saved as text files. The text files were then imported into Matlab (Mathworks, Natick, MA, USA) and analysed with custom-written scripts that calculated the angle of the line formed by the centroids of the eyes relative to an external but arbitrary reference. This procedure allowed determination of the head angle in space over time during a swimming episode (Fig. 1A,C). The timing was extracted from the time stamp assigned to each frame by the camera from its internal 8 kHz cycler (see above). The head angle trace was smoothed using a third-order polynomial Savitzky–Golay filter based on seven consecutive time points (Fig. 1C). The smoothed trace was then used to calculate the angular velocity by dividing the difference in head angle between two frames by the time difference. This procedure was repeated to calculate the angular acceleration. To calculate forward velocity, the distance covered between subsequent frames by the interocular midpoint was determined and divided by the time difference between the two frames. The Reynolds number (Re) was calculated as $Re=U\times L/\nu$, where U is forward velocity in mm s^{-1}, L is total length of the animal and ν is kinematic viscosity of water at 17°C (1.0811 mm^2 s^{-1}; see http://www.viscopedia.com/viscosity-tables/substances/water/). The frequency and amplitude of the head oscillations during each swimming episode were calculated based on the left–right alternating peaks of the head angle trace. The amplitude between the peak in one direction and the next peak in the other direction was determined as the half-cycle amplitude (i.e. one right–left or left–right movement of the head), and the corresponding duration was determined as the time required for half a swimming cycle period. From this half-period, we calculated the instantaneous head oscillation frequency, which directly corresponds to the tail-beat frequency, as the head and trunk are tightly coupled (Chagnaud et al., 2012), and will be referred to as swimming frequency throughout the study.

From over 400 swimming episodes without wall touch, selected episodes were further analysed based on the criterion of at least six consecutive head oscillations (i.e. half-cycles) with an amplitude between 4 and 90 deg, to exclude image jitter as well as very large turns such as escape-related C-starts. These selected episodes from the same animal were pooled, and the median and interquartile ranges for each animal are reported.

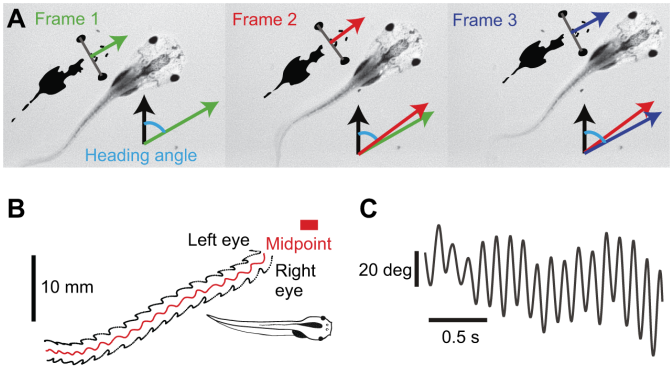

Fig. 1. Extraction of parameters of head movement kinematics. (A) Sequence of three video frames separated by ∼80 ms, depicting the dorsal view of a stage 50 tadpole during free swimming along with the thresholded black-and-white image of the animal as an inset. From the thresholded image, the position of the eyes was extracted, and the angle of the heading direction was calculated (coloured forward arrow). (B) Trajectories of the two eyes (black dots) and interocular midpoint (red), obtained from a video recording at 200 frames s^{-1} of a 2 s swim episode in a stage 48 tadpole. Tadpole scheme from Hänzi and Straka (2016a). (C) Head angles calculated from the trajectory in B, indicating very regular left–right head oscillations during swimming.

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Anatomical measurements

To correlate the extent of morphological changes with the different developmental stages, the following parameters were extracted from the videos: distance between the eyes (interocular distance), total length of the animal, trunk length, and tail width at the level of the hindlimbs. The interocular distance was determined as the median of the distance between the two eyes in all frames of a particular animal. The remaining parameters were manually extracted using Fiji based on two frames each from two different video recordings of the same animal. The final value was then calculated as the mean of these four values.

Fictive swimming: electrophysiology

To compare the frequency of free swimming with fictive swimming – which excludes biomechanical properties of tail motion as well as the corresponding proprioceptive feedback – multi-unit ventral root spiking activity was recorded in stationary semi-intact *in vitro* preparations of larvae at different developmental stages. Some of the data that were included in the present study (13 out of 18 animals) were obtained in the framework of a previous study (Hänzi et al., 2015). Semi-intact preparations were obtained based on the procedure described previously (Hänzi et al., 2015). Briefly, tadpoles were anaesthetised using 0.05% 3-aminobenzoic acid ethyl ester (MS-222; Sigma-Aldrich, UK), decapitated and decerebrated in ice-cold Ringer solution (composition in mmol l^{-1}: NaCl, 75; KCl, 2; $CaCl_2$, 2; $MgCl_2$, 0.5; $NaHCO_3$, 25; glucose, 11; pH 7.4). The spinal cord was exposed approximately from segment 2 to segment 15, with the remaining part of the tail left intact. The ventral roots along the exposed spinal cord were cut, the trunk muscles removed, and the preparation was transferred into the recording chamber, where it was continuously superfused with freshly oxygenated Ringer solution at a rate of about 1–3 ml min^{-1} while the temperature was kept at 17±0.5°C. One or more ventral roots of spinal segments 8–15 were recorded using glass suction electrodes (pulled on a P-97 Brown/Flaming puller, Sutter Instruments, Novato, CA, USA) with the tip individually adjusted to the size of the nerve roots. Fictive swimming occurred either spontaneously or following gentle touch of the caudal part of the tail or the otic capsule. Signals were amplified (EXT 10-2F; npi electronics, Tamm, Germany), digitised at 10 kHz (CED 1401, Cambridge Electronic Design, Cambridge, UK), recorded using Spike2 (Cambridge Electronic Design) and stored for subsequent off-line analysis.

Episodes of fictive swimming, identified by rhythmic ventral root activity (Combes et al., 2004), were exported from Spike2 into Matlab; episodes of fictive struggle or potential escape responses, indicated by strong concurrent bilateral and single alternating bursting, were excluded from further analysis. The spike discharge was extracted using custom-written scripts based on code written by Daniel Wagenaar (http://www.its.caltech.edu/~daw/teach.html#matlab). Briefly, spikes were extracted using a threshold based on the noise level in the recording trace (5 times the root mean square) and bursts were defined as at least two spikes with maximal inter-spike intervals of 20–70 ms. The swimming frequency was then determined as the inverse of the inter-burst interval within one ventral root.

Statistics

Statistical analysis was carried out in Matlab using custom-written scripts. Distributions were tested for normality using the Shapiro–Wilk test; if significant, the appropriate non-parametric test was used. To test for changes across development, a regression of the total length of the animal to the median value per animal was calculated for each kinematic parameter. $P<0.05$ was considered significant.

RESULTS

Increase of body size during development

The post-embryonic, larval development in amphibians is generally characterised by a considerable increase in body size (Fig. 2A; also see Nieuwkoop and Faber, 1956). Determination of body and trunk length, interocular distance and tail width at the level of the emerging hindlimbs (Fig. 2B) revealed that these parameters increased steadily and correlated with developmental stage (N=35; Fig. 2C–F) previously used to describe the progressive steps of *X. laevis* ontogeny (Nieuwkoop and Faber, 1956). The size of the tadpoles (total body length) employed in the current study ranged between ∼10 mm for the youngest (stage 46) and ∼45 mm for the oldest animals (stage 56; Fig. 2C). This parameter reliably predicted the developmental stages of *Xenopus* tadpoles (Fig. 2C). This prediction was equally well achieved with the other measured parameters such as trunk length (Fig. 2D), interocular distance

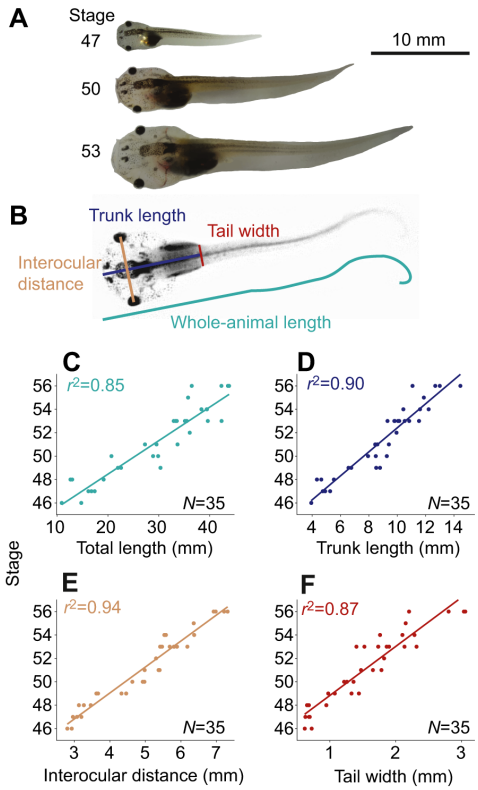

Fig. 2. Tadpole growth. (A) Images of *Xenopus* tadpoles on the same scale, with the developmental stage indicated (modified from Hänzi and Straka, 2016b). (B) Illustration of the anatomical parameters, extracted from a video frame of a stage 51 animal (see Materials and methods for details). (C–F) Developmental changes of the total length of the animal (C), trunk length (D), interocular distance (E) and tail width at the position where the hindlimbs emerge (F) in a total of 35 animals. Note the high r^2 values of linear regressions of all parameters with respect to developmental stage.

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(Fig. 2E) and tail width (Fig. 2F). This was surprising, given the fact that developmental stages in *X. laevis* tadpoles were defined on the basis of multiple morphological features including the progression of limb growth and differentiation or the size and visibility of visceral organs (Nieuwkoop and Faber, 1956). Even though all extracted anatomical parameters were able to predict developmental advancement, we used total body length as linear regressor, thereby facilitating comparisons with other studies.

Kinematic parameters

Video tracking of tadpoles (Fig. 1A) was used to extract the position of both eyes and thereby to determine the trajectory of the animal during episodes of free swimming (Figs 1B, 3A). These data on eye position served to calculate the angle of the head over time relative to an external but arbitrary reference (Figs 1C, 3B). From the excursions of the head angle, the amplitude of the head oscillations was determined (Fig. 3D, indicated as amplitude), as well as the 'instantaneous' swimming frequency (Fig. 3C). Furthermore, angular acceleration and velocity were calculated from the head angle (Fig. 3E,F) using the time stamp of each frame as precise timing information. Finally, from the interocular midpoint (red in Fig. 3A) and the timing information, the animal's forward velocity was determined (Fig. 3G). The two examples of differently sized animals (stages 48 and 54) shown in Fig. 3 indicate that the swimming performance of smaller larvae differs considerably from that of larger animals in some of the kinematic parameters (trajectories on the left and right of Fig. 3A are shown on the same spatial scale; see also Movies 1 and 2). Notably, the head/tail oscillations during an example swimming episode of a young tadpole (13 mm, stage 48, green) generally occurred at higher frequencies (Fig. 3C) compared with those of an older animal (38 mm, stage 54, blue). Accordingly, swimming in the younger tadpole is characterised by higher angular velocity and acceleration (Fig. 3E,F). However, the maximal forward velocity was similar despite the differences in body size (Fig. 3G).

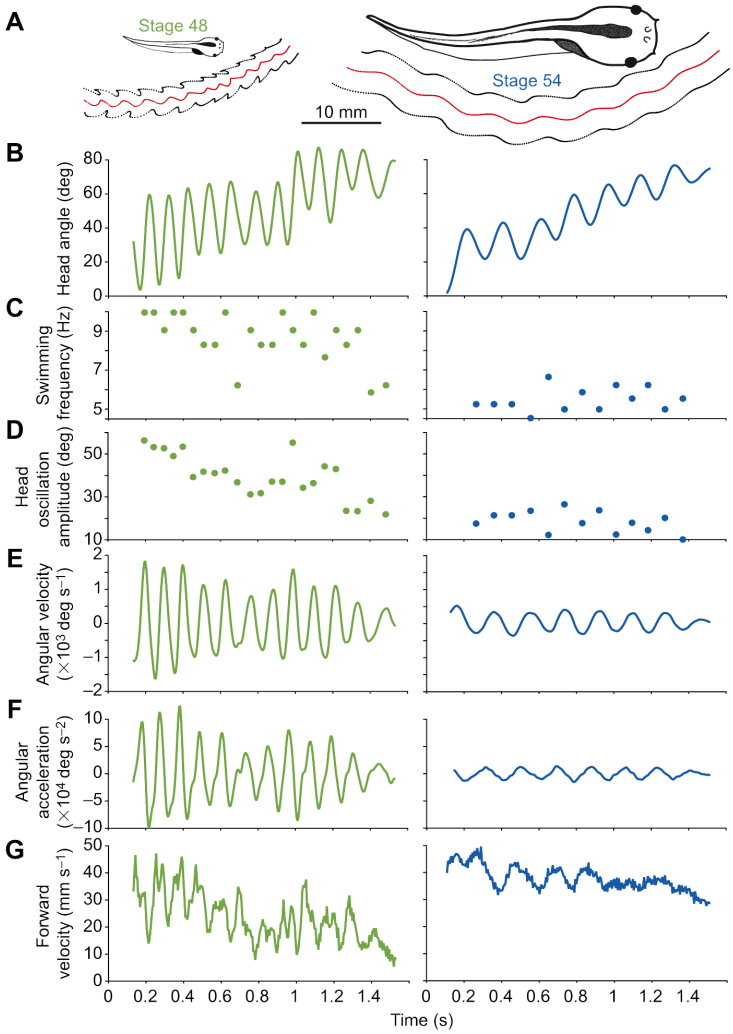

Fig. 3. Examples of swimming episodes of two differently sized larvae along with extracted kinematic parameters. (A) Swimming trajectories of a 13 mm-long stage 48 (left) and a 38 mm-long stage 54 (right) tadpole over a period of 1.4 s. Eye positions (black trajectories) and interocular midpoints (red trajectory) were obtained from video recordings at 200 frames s^{-1}. These sequences were obtained from Movies 1 and 2. The trajectories and animal drawings are shown on the same scale (drawings from Hänzi and Straka, 2016a). (B) Angle of the head over time extracted from the trajectories shown in A. (C,D) Swimming frequency (C) and amplitude (D) of head oscillations over time calculated from the head angle oscillations shown in B. (E,F) Angular velocity (E) and acceleration (F) of the head over time during the swimming episodes shown in A, calculated from smoothed head angle (B) and velocity (E) traces, respectively (see Materials and methods). (G) Forward velocity calculated from the distance covered by the interocular midpoint during the swimming episodes shown in A.

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Consistency of kinematic parameters over repeated swimming episodes

Episodes of free swimming for quantitative analysis were selected based on two criteria: first, the animal did not touch a vertical wall and second, at least six consecutive half-cycles of swimming (i.e. left–right or right–left head movements) were within an amplitude range between 4 and 90 deg (see Materials and methods for details). The consistency of the kinematic parameters over multiple swimming episodes within the same animal was tested for those parameters that were extracted on the basis of each half-cycle. The frequency and amplitude distribution of the 15 swimming episodes of a 13 mm stage 48 tadpole are shown in Fig. 4A,C, with the different episodes plotted on the *y*-axis and the half-cycles within each episode represented as bars on the *x*-axis. The presence of mostly blue–green bars in the colour-coded representation (Fig. 4A) indicated that the swimming frequency within a given episode as well as between different episodes was relatively consistent. In contrast, the amplitude of the head oscillations varied considerably both within and between episodes, as implied by the widely different colours within and between episodes (Fig. 4C).

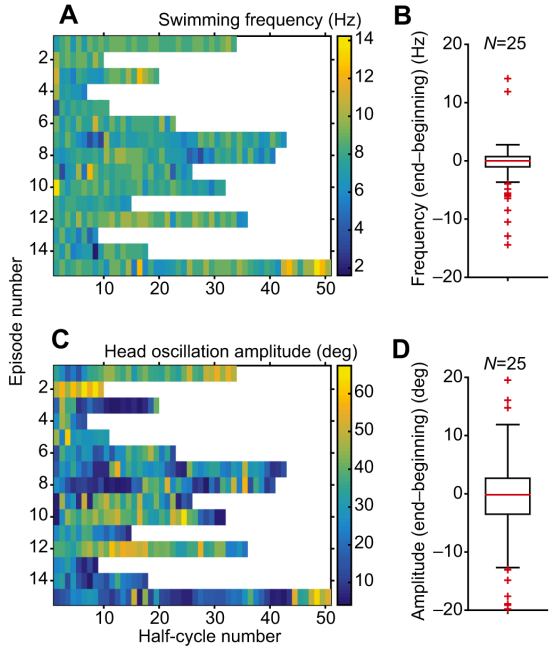

Fig. 4. Intra-animal variability of swimming frequency and head oscillation amplitude. (A,C) Colour-coded swimming frequency (A) and head oscillation amplitude (C) across 15 episodes (*y*-axis) in a 13 mm stage 48 tadpole, with the number of half-cycles indicated on the *x*-axis. The relatively uniform colour distribution in A indicates little intra- and inter-episodal variability of the swimming frequency, whereas the large variation of the colour in C shows a considerable intra- and inter-episodal variability of head oscillation amplitude in this typical animal. (B,D) Boxplot depicting the difference in the mean frequency (B) and the mean head oscillation amplitude (D) of the first and last two half-cycles of a given episode (221 episodes from *N*=25 animals). Note the spread of differences either side of zero in B and D, indicating that the distribution of differences between the beginning and end of the episode is not significantly different from zero (one-sample Wilcoxon signed-rank tests; the *y*-axis was cropped for both plots) and, thus, the frequency and amplitude of swimming were very similar at the beginning and end of each swimming episode.

As previous reports on swimming *X. laevis* hatchlings reported that the frequency of the head/tail oscillations decreased gradually over the course of a given swimming episode (Kahn et al., 1982; Sillar and Roberts, 1993; Sillar et al., 1991), the difference between the mean frequency of the first two and the last two half-cycles of each episode was calculated (Fig. 4B; 221 episodes in N=25 animals) and found not to be significantly different from zero (one-sample Wilcoxon signed-rank test, P>0.05). Similarly, the difference between the amplitude of the head oscillation at the beginning and the end of each swim episode was not statistically different from zero either (Fig. 4D; 221 episodes in N=25 animals, one-sample Wilcoxon signed-rank test, P>0.05).

Changes in kinematic parameters with development

To compare the kinematic parameters of swimming in animals with progressively larger total body length, all episodes for a given animal were pooled, and the median and interquartile ranges per animal (N=25) were plotted. The largest change was observed for the frequency of swimming, which decreased considerably with growth (Fig. 5A; regression of total length of the animal against its median swimming frequency: r^2=0.77, P<0.0001). In contrast, the amplitude of the head oscillations was relatively variable within each animal (see Fig. 4), and the median amplitude remained relatively constant across the differently sized animals (Fig. 5B; regression of total length against median amplitude, P>0.05). The median angular velocity and acceleration during swimming in the smallest animals were remarkably high: ∼150–600 deg s^{-1} and ∼5000–20,000 deg s^{-2}, respectively. As angular velocity and acceleration derive from the combination of frequency and amplitude of the head oscillations, the decreasing frequency with development led to a decrease of the angular head velocity and acceleration with growth (Fig. 5C,D; regression of total length against median angular velocity: r^2=0.24, P=0.01; regression of total length against median angular acceleration: r^2=0.43, P=0.0004). In small larvae, the maximal values for head velocity and acceleration reached ∼2500 deg s^{-1} and ∼250,000 deg s^{-2}, respectively; in contrast, in larger animals, the maximal values were ∼1000 deg s^{-1} for angular velocity and ∼50,000 deg s^{-2} for angular acceleration.

In contrast to the developmental changes in angular head motion parameters, the absolute forward velocity during swimming was comparable between smaller and larger animals (Fig. 5E; regression of total body length against median forward velocity in mm s^{-1}, P=0.06). However, when calculating swimming speed with respect to body length, smaller animals swam faster than larger larvae (Fig. 5F; regression of total body length against median forward velocity in body lengths per second, r^2=0.28, P=0.006). Based on the forward velocity and the size of each animal, *Re* was calculated (as total length multiplied by forward velocity in mm s^{-1} divided by the kinematic viscosity of water at 17°C; see Materials and methods). The *Re* values ranged from ∼150 for slow-swimming small larvae to ∼1500 for fast-swimming large tadpoles with a gradual size-related increase (data not shown; regression of total body length against median *Re*: r^2=0.64, P<0.0001). Only 4 out of 25 animals had a median *Re*<200, which is considered by some authors to be the boundary between intermediate and inertial hydrodynamic regimes (Fuiman and Webb, 1988; but see also McHenry and Lauder, 2005).

Comparing free and fictive swimming

To determine the relationship between intrinsically generated locomotor commands in the absence of sensory feedback and actual tail oscillations during free swimming, a set of *in vitro*

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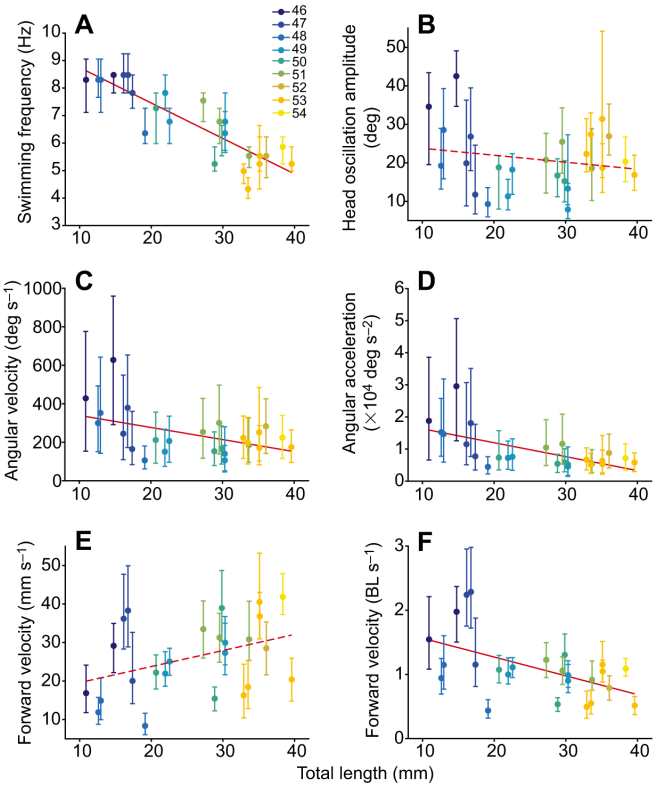

Fig. 5. Changes in kinematic parameters over development. (A–F) Relationship between total body length of the animal and swimming frequency (A), head oscillation amplitude (B), angular velocity (C), angular acceleration (D) and forward velocity in mm s^{-1} (E) and body lengths (BL) s^{-1} (F). All plots show the median and interquartile range obtained for each of N=25 animals; each animal is colour coded according to its developmental stage (key shown in A). The regression of total body length against these kinematic parameters was significant in A (r^2=0.77, P<0.0001), C (r^2=0.24, P=0.01), D (r^2=0.43, P=0.0004) and F (r^2=0.28, P=0.006), but not in B and E, indicating that swimming frequency, angular velocity and acceleration decrease with development, head oscillation amplitude and absolute forward velocity remain unchanged, while the forward velocity in BL s^{-1} decreases. Note the relatively small intra-individual variability of swimming frequency (A).

experiments on semi-intact preparations (Fig. 6A) was conducted. In these experiments, we recorded episodes of fictive swimming, consisting of locomotor activity in the absence of muscle contractions and sensory inputs (see Combes et al., 2004). The bilaterally alternating bursts in ventral roots on both sides recorded in isolated preparations would cause left–right alternating contractions of the axial muscles in the intact animal. Such sequences of fictive swimming, extracellularly recorded as rhythmic burst discharge of ventral roots at the level of spinal segments 8–15, are shown in Fig. 6B. To evaluate temporal changes in the burst rhythm during larval growth, we recorded ventral root spike activity in tadpoles of different sizes (15–45 mm, developmental stages 47–54, N=17). Examples of ventral root recordings in three animals with body lengths of 18, 30 and 42 mm corresponding to developmental stages 47, 50 and 53, respectively, are shown in Fig. 6B. These typical examples revealed a gradual decrease in the burst rhythm frequency with increasing body size. The burst rhythm of larger animals had a lower frequency (see traces on the right of Fig. 6B). In addition, it appeared that individual bursts in smaller animals consisted of fewer spikes compared with those in larger tadpoles, probably due to the presence of more active motoneurons in the larger animals (right trace in Fig. 6B). For quantification, the median burst frequency was determined for each animal (blue circles in Fig. 6C; red encircled dots represent data from the larvae depicted in Fig. 6B) and was plotted along with the frequency of free swimming (black circles in Fig. 6C, data were obtained from Fig. 5A) against body length. In general, the frequency of fictive and free swimming was relatively similar for animals of a given size. In addition, the frequency decreased markedly with body growth both for fictive swimming (Fig. 6C, blue; regression of body length against median fictive swimming frequency: r^2=0.46, P=0.003) and for tail oscillations during free swimming (Fig. 6C, black; slope of best fit for body length against median frequency was −0.13 for free swimming and −0.1 for fictive swimming). However, the intercept exhibited a lower value for fictive swimming (10.03 for free swimming and 8.7 for fictive swimming, Fig. 6C; also compare dashed and solid lines in Fig. 6D; see Discussion).

DISCUSSION

Swimming in *X. laevis* tadpoles is produced by horizontal undulatory tail movements. The tight anatomical connection between the head/body and the tail in these animals causes a strict coupling between axial muscle-driven tail undulations and head oscillations. During the post-embryonic, larval development, when animal length increases ∼3-fold, the swimming frequency concurrently decreases ∼2-fold. As a consequence, both angular velocity and acceleration decrease considerably with larval development, thereby diminishing the dynamic range of angular head motion-related vestibular signals. These results will be compared with those of previous studies on swimming kinematics and developmental changes in *Xenopus* and other species.

Swimming kinematics

Swimming in *Xenopus* tadpoles occurs in two major forms with respect to generated forward velocity and motion consequences for the vestibular organs. Low-speed swimming or cruising is generated by undulations of the most caudal part of the tail, which causes only

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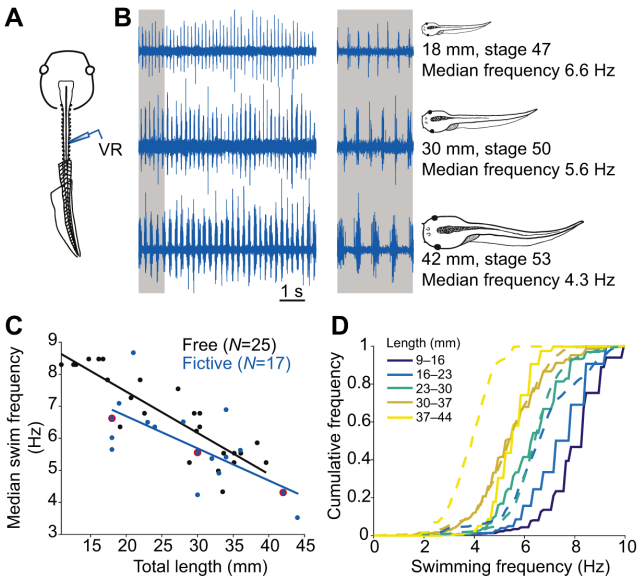

Fig. 6. Comparison of frequencies in free and fictive swimming. (A) Schematic diagram of an isolated, stationary *in vitro* preparation of a tadpole with a suction electrode for recording from a ventral root (VR). (B) Examples of fictive swimming episodes, indicated by rhythmic ventral root discharge, in three differently sized animals: 18 mm (stage 47, top), 30 mm (stage 50, middle) and 42 mm (stage 53, bottom). The first 1 s of swimming is plotted on an extended time scale on the right (grey box), and the median swimming frequency over all episodes of the respective animal is indicated. Tadpole schemes are shown on the same spatial scale (from Hänzi and Straka, 2016a). (C) Relationship between the median frequency of free and fictive swimming and total body length of the animal (the free-swimming data derived from Fig. 5A). Lines show best linear fits, with very similar slopes for free (–0.13, r^2=0.77) and fictive (–0.1, r^2=0.46) swimming. The data for the three animals shown in B are encircled in red. (D) Empirical cumulative distribution of swimming frequency for freely (solid lines; for details on the calculation of instantaneous frequency from the discrete frames, see Material and methods) and fictively swimming preparations (dashed lines). Animals were pooled into five groups according to their body length. Note that the darker traces (indicating smaller animals) are more to the right, in the higher frequency range.

minimal forward thrust and virtually no cyclic left–right head undulations (Hoff and Wassersug, 1986); this mode has also been called ‘sculling’ and probably serves to hold the vertical position in the water column, as these tadpoles are positively buoyant. In contrast, high-speed swimming is produced by undulations of the entire tail and generates considerable forward velocity as well as cyclic head oscillations, which form angular acceleration stimuli for activating the horizontal semicircular canals. This fast swimming mode, with considerable consequences for vestibular sensation, was examined in the current study. At first glance, this locomotor style seems relatively inefficient, because it appears non-streamlined, but it is exactly this lateral undulatory motion that generates forward thrust (Liu et al., 1997). In the absence of a flexible neck in most fish and tadpole-like amphibian larvae, propulsive swimming-related tail and head movements are tightly coupled and thus allow swimming kinematics to be inferred from head movements (Chagnaud et al., 2012). Moreover, our combined analysis of swimming kinematics and concurrent head motion provided the necessary quantitative information to interpret developmental changes with regard to locomotor dynamics and motion detection capabilities by the vestibular system.

Forward speed is one of the main parameters when describing locomotor behaviour and dynamics. The tadpoles in the current study mostly swam with an absolute speed of 10–60 mm s^{-1}, largely independent of developmental stage, although larger animals generally tended to be slightly faster (Fig. 5E). This magnitude is comparable to the forward speed observed shortly after hatching (45–61 mm s^{-1}; Kahn et al., 1982) as well as to the speed of 6–9 day old zebrafish larvae (Budick and O’Malley, 2000), suggesting that aquatic fish/tadpole-like animals with similar size and morphological shape have comparable locomotor characteristics. However, because of the considerable growth of the body during larval development in *Xenopus*, the forward speed relative to body length decreases from ∼1.5 to ∼0.5 body lengths per second (BL s^{-1}). This is in marked contrast to a previous study on *Xenopus* tadpoles, in which a swimming speed of around 6 BL s^{-1} was reported (Hoff and Wassersug, 1986). Unfortunately, the small number of animals in that study (*N*=4) and the absence of a description of either developmental stage or size makes it difficult to compare and interpret the noticeable difference from the results of our study.

Swimming frequency is a locomotor parameter of fish and aquatic amphibian locomotor activity that has been widely analysed and thus allows a comparison within and across species. In our study, swimming frequencies of *Xenopus* larvae ranged from 4 to 10 Hz across tadpoles of 10–45 mm, corresponding to larval stages 46–56, and decreased significantly with development. As younger tadpoles below stage 46 (smaller than 14 mm) swim with even higher frequencies shortly after hatching (10–25 Hz; Kahn et al., 1982), this progressive developmental reduction in frequency between hatching and metamorphic climax appears to be a general feature of larval ontogeny. While larval *Xenopus* swim with little frequency variation at each developmental stage, larval zebrafish can express either slow swimming, with frequencies of 25–40 Hz, or burst-like swimming, with frequencies of 45–75 Hz (Budick and O’Malley, 2000). Accordingly, the swimming of *Xenopus* tadpoles is more comparable to the slow swimming mode of larval zebrafish. Swimming frequency is also the parameter that can most easily be compared between free and fictive swimming. This has been done in larval bullfrogs (Stehouwer and Farel, 1980), newt embryos (Soffe et al., 1983), larval angelfish (Yoshida et al., 1996), zebrafish (Lambert et al., 2012b) and young *Xenopus* tadpoles, and in all these cases, free and fictive swimming were found to be at least qualitatively similar. Our current findings add to these previous reports that the swimming frequency is also quantitatively similar between free and fictive swimming, with a gradual developmental decrease of the swim rhythm.

Developmental changes in locomotor patterns

The progressive alteration of the swimming kinematics as the larvae grew from 10 to 45 mm was substantiated by results of previous studies on even younger tadpoles of *X. laevis*. While mostly attached to a substrate between stages 37 and 38 (Boothby and Roberts,

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1992; Jamieson and Roberts, 2000), the propensity of *Xenopus* larvae to spontaneously swim freely increases after the onset of active feeding at stage 45 (Nieuwkoop and Faber, 1956), as confirmed by a developmental study on fictively swimming animals (Currie et al., 2016). At this time, the frequency of the tail undulations is generally high (10–25 Hz; Kahn and Roberts, 1982; Kahn et al., 1982) and each swimming bout is characterised by decreasing frequencies over the course of the episode (Kahn et al., 1982; Sillar and Roberts, 1993; Sillar et al., 1991). This contrasts with the observation in the older and thus larger tadpoles studied here, where the swimming frequency remains largely constant throughout a given episode (Fig. 4A,B), suggesting that the central pattern generator in the spinal cord at this developmental stage is capable of maintaining a relatively stable rhythm over time. With further developmental progress, the swimming frequency drops fairly linearly (Fig. 5A) to values of 2–4 Hz just before metamorphic climax (Combes et al., 2004; von Uckermann et al., 2016). A likely explanation is that this low tail undulation frequency towards the end of the larval period may derive from the gradually stronger phase coupling of the axial swim rhythm with the concurrently maturating hindlimb kick propulsion that occurs at a lower frequency (Rauscent et al., 2006) and finally replaces the tail-based undulatory swimming as the major locomotor strategy (Combes et al., 2004).

Comparable post-embryonic changes in the frequency of locomotor patterns have also been described in other animals. In rats, the limb movement frequency – tested in water to avoid effects of weak limb musculature – increases during the first 2 weeks after birth (Bekoff and Trainer, 1979); in chickens, the wing-beat frequency increases during the 2 weeks post-hatching (Provine, 1981a); in locusts the wing-beat frequency increases after the final moult (Altman, 1975; Kutsch, 1971, 1974); in moths, the frequency increases before the adult stage is reached, even in the absence of an overt behaviour (Kammer and Kinnamon, 1979). In chickens, the increase starts before and continues after the behaviour (flight) becomes effective (Provine, 1981a). In locusts, the situation is most similar to amphibian tadpoles in the sense that the frequency changes occur after the behaviour (flight/swimming) is implemented (Kutsch, 1971). The increase in locomotor frequency reported in all these examples contrasts with the reduction in tadpole swimming frequency in our study. Nonetheless, all alterations are probably adaptations to optimise or at least maintain locomotor efficiency. Accordingly, the decrease in swim frequency with concurrently increasing body length of the tadpoles might be due either to constraints that the aquatic environment imposes on the swimming of fish-like vertebrates or to the growth-related increase in body rigidity that impairs the execution of flexible tail undulations (Sfakiotakis et al., 1999). In fact, compatible with our results, larger individuals of a given fish species express a slower swim rhythm compared with smaller specimens (Bainbridge, 1957), suggesting that the developmental decrease of the swim rhythm in *Xenopus* tadpoles represents a general feature of growing aquatic vertebrates.

The question arises as to what causes these developmental changes in locomotor frequency. For swimming, hydrodynamic requirements might play a crucial role. For instance, for larval and adult anchovy, which have ‘grown out’ of the viscous hydrodynamic regime, an intermittent beat-and-glide swimming is more efficient than continuous swimming (Weihs, 1980). This example shows a change in locomotion performance that is related to a change in hydrodynamic regime. Similarly, for larval and juvenile zebrafish, the aquatic environment probably represents a rather viscous regime, whereas adult swimming operates in an inertial regime (McHenry and Lauder, 2005). For tadpoles, Liu et al. (1996) determined a Reynolds number of 7200 for ranid tadpoles with a length of 47 mm at a swim speed of 5 BL s^{-1}. At variance with the locomotor performance of the relatively large and fast amphibian larvae in that study, the generally smaller *Xenopus* tadpoles in our study (15–45 mm) swam at a slower speed, corresponding to a Reynolds number of 300–1500, which is more comparable to that reported for the similarly sized older larvae and adult zebrafish (McHenry and Lauder, 2005). These authors also used body length to calculate *Re*, whereas other authors used a diameter measure for ranid tadpoles (Dudley et al., 1991). A direct comparison of Reynolds numbers obtained in different species is therefore difficult and only allows an approximate inference of the respective locomotor regime (Vogel, 1996). Moreover, different authors define the hydrodynamic regimes differently (Liu et al., 1996; McHenry and Lauder, 2005). Nevertheless, we expect that the vast majority of the tadpoles in our study, with the exception of the smallest larvae, experienced a similar physical regime. Assuming that the most pronounced hydrodynamic changes of locomotor performance in *Xenopus* larvae, as in developing zebrafish, occur at animal lengths of 5–15 mm (Fuiman and Webb, 1988), it is unlikely that hydrodynamics as a critical parameter for aquatic locomotion is the sole driving force for the observed changes in swimming kinematics in the larger tadpoles of our study.

The more general question remains of whether these developmental changes in the frequency of centrally generated locomotor patterns are driven by sensory feedback. In tadpoles, removal of online sensory feedback, as during fictive swimming, slightly reduces the frequency (Fig. 6A); however, this effect is very small compared with the overall developmental decrease. Therefore, the decrease in swimming frequency with increasing body size is a long-term adaptation (according to the terminology of Pearson, 2000). This contrasts with the situation in Australian plague locusts, where the fixation of the wings leads to a frequency similar to that before the developmental change started (Altman, 1975). Sensory feedback in the locust case thus plays an important role on a short time scale. However, this differs on a longer time scale, i.e. the 3 weeks over which the frequency changes, indicating that no practice or sensory feedback is required for the long-term changes to occur (Altman, 1975). This is very similar to the situation in chickens, where the increase in wing flapping over the first 2 weeks post-hatching occurs with featherless wings or wings that were immobilised during those 2 weeks (Provine, 1981a,b) and even in the absence of wings with only wing stumps (Provine, 1979). While these interventions do not abolish sensory feedback completely, they at least suggest that practice of the movements with its associated self-generated sensory feedback is not necessary for the developmental change to occur. Because removal of sensory feedback over a longer time scale is very difficult to achieve in tadpoles, any inference on the impact of such signals on the locomotor output rhythm must necessarily be speculative. The spinal oscillator frequency might change intrinsically without any influence of sensory feedback, or the central effect of sensory feedback might change, or the nature of the sensory feedback might change with development. These possibilities are not mutually exclusive as changes are likely to occur at multiple levels. For instance, the nature of the feedback that the tadpole spinal cord receives directly from the trunk changes over the course of development, from only light touch mediated through Rohon–Beard cells (Roberts and Hayes, 1977) to more extensive proprioceptive feedback via the dorsal roots (Hughes, 1957; Nieuwkoop and Faber, 1956).

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Consequences of swimming-related head oscillations for vestibular motion detection

The undulatory swimming at all larval stages caused oscillatory head movements with a considerable velocity/acceleration component that forms an adequate stimulus for activating responses in hair cells of the horizontal semicircular canals. The peak angular velocities of head movements that occur during tadpole swimming in the current study exceeded those reported for bucking cattle and spinning dolphins, which are in the range 200–600 deg s^{-1} (Kandel and Hullar, 2010). However, animals that have a body size closer to that of *Xenopus* tadpoles also experience very high angular velocities and accelerations during swimming. In fact, the peak angular velocity generated during undulatory swimming in larval or adult zebrafish reaches up to 10,000 deg s^{-1} (Fontaine et al., 2008) and thus is approximately an order of magnitude larger than that observed during swimming in *Xenopus* tadpoles throughout most of the pre-metamorphic period. During routine swimming, larval zebrafish reach an angular velocity of 4000–10,000 deg s^{-1} and up to 32,000 deg s^{-1} during escape responses (Budick and O'Malley, 2000). Most of the difference in the magnitude of swimming-related head dynamics between *Xenopus* and zebrafish is probably due to the substantially higher swimming frequency in the latter species, which reaches up to 70 Hz (see above). As larval *Xenopus* hatchlings also swim with a higher frequency than older and thus larger larvae (Sillar et al., 1991), it is likely that higher angular velocities are regularly reached.

Detection of head movements by the vestibular system depends both on the magnitude of the head movements and on the overall sensitivity of the sensory structures. The sensitivity of semicircular canals depends on sufficiently large lumen and circuit radii to allow the inertial forces to generate an acceleration-induced and endolymph-mediated cupula displacement (Muller, 1999). This is particularly critical in vertebrates, which develop through small-sized larvae such as fish and amphibians. In fact, the horizontal angular vestibulo-ocular reflex (VOR) in *Xenopus* tadpoles has a relatively late ontogenetic onset (stage 48) that depends on the acquisition of a sufficiently large semicircular canal lumen diameter (Lambert et al., 2008), a finding that also applies to zebrafish (Beck et al., 2004). The interpretation of the physiological findings depended on the assumption that natural angular head movements in *Xenopus* tadpoles occur largely within the range of the experimentally employed horizontal accelerations and do not exceed 400 deg s^{-2} (Lambert et al., 2008). However, the much higher angular accelerations during free swimming in the current study would allow the detection of self-generated head movements with even smaller semicircular canals, suggesting an earlier functional onset of the angular VOR in *Xenopus* tadpoles. Accordingly, the detection of swimming-related horizontal head oscillations and VOR-driven gaze stabilisation might therefore occur immediately after semicircular canal formation at stage 46 (Haddon and Lewis, 1991). However, at least in older animals (stage 55), a locomotor efference copy suppresses the signals from the horizontal semicircular canals during swimming (Chagnaud et al., 2015; Lambert et al., 2012a). This mechanism might already be implemented at younger stages, compatible with the necessity of efference copy-mediated adjustments of the sensory sensitivity, including a considerable attenuation of the sensory inputs. Thus, even though young *Xenopus* larvae generate very high angular accelerations during self-motion, these stimuli might be ineffective for activating an angular VOR, thereby maintaining the overall validity of the earlier study on the ontogeny of gaze stabilisation (Lambert et al., 2008).

Conclusions

Our results show that individual *Xenopus* tadpoles swim with a relatively constant frequency, while the amplitude of the head oscillations is rather variable. During development, the average amplitude of the swimming-related head oscillations remains similar, whereas the frequency decreases as tadpoles grow. Accordingly, this causes a decrease of the angular head velocity and acceleration in older larvae. Surprisingly, younger animals swam faster than older animals if the speed was expressed in terms of body length. Finally, the similarity of free and fictive swimming frequency in tadpoles of a given size along with the concurrent developmental decrease of swimming frequency under both experimental conditions suggests a dominant of the central pattern generator in determining the swim rhythm.

Acknowledgements

The authors thank Dr Larry Hoffman for initiating these experiments and for valuable discussions of the data.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Investigation, Software, Visualization: S.H.; Supervision, Project administration, Funding acquisition: H.S.; Conceptualization, Writing: S.H. and H.S.

Funding

This study was funded by the Deutsche Forschungsgemeinschaft (STR 478/3-1) and the Bundesministerium für Bildung und Forschung (grant number 01 GQ 1407).

Data availability

Data are available from figshare at https://figshare.com/s/e9e7d4dc6b78c72f1151.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.146449.supplemental

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4 Motor consequences of swimming

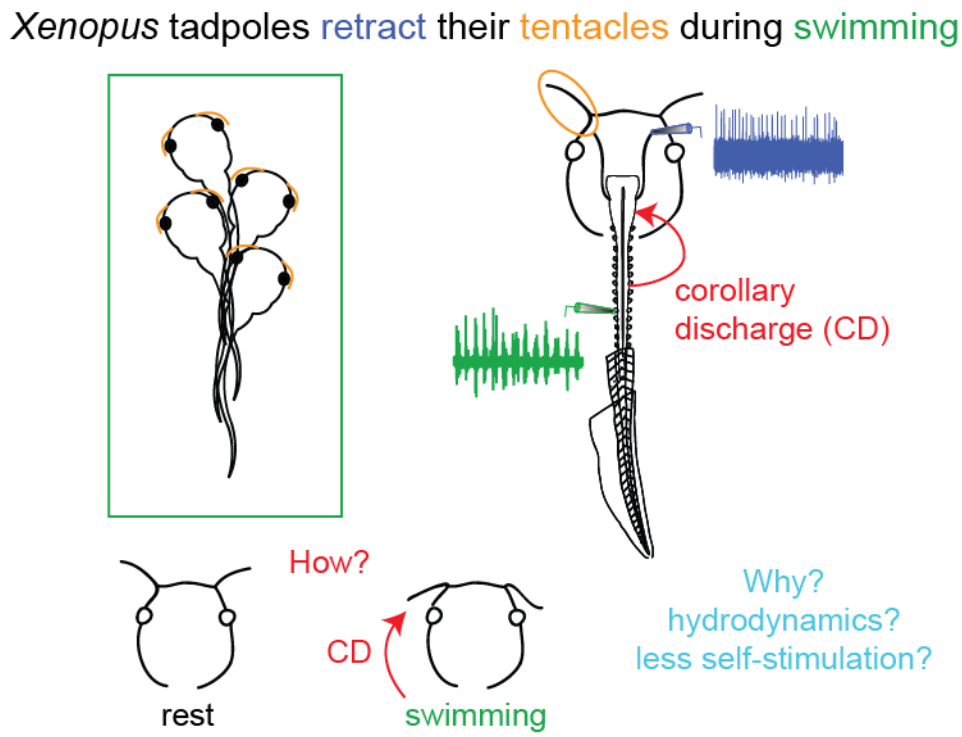

Figure 6. Graphical abstract for the study on tentacle retraction during swimming.

4.1 Citation

Hänzi, S., Banchi, R., Straka, H., and Chagnaud, B.P. (2015). Locomotor corollary activation of trigeminal motoneurons: coupling of discrete motor behaviors. J. Exp. Biol. *218*, 1748–1758.

4.2 Contributions

S.H., R.B., H.S. and B.P.C. planned the experiments, S.H., R.B., and B.P.C. acquired and analyzed the data, S.H., R.B., H.S. and B.P.C. wrote the manuscript.

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RESEARCH ARTICLE

Locomotor corollary activation of trigeminal motoneurons: coupling of discrete motor behaviors

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ABSTRACT

During motor behavior, corollary discharges of the underlying motor commands inform sensory-motor systems about impending or ongoing movements. These signals generally limit the impact of self-generated sensory stimuli but also induce motor reactions that stabilize sensory perception. Here, we demonstrate in isolated preparations of *Xenopus laevis* tadpoles that locomotor corollary discharge provokes a retraction of the mechanoreceptive tentacles during fictive swimming. In the absence of sensory feedback, these signals activate a cluster of trigeminal motoneurons that cause a contraction of the tentacle muscle. This corollary discharge encodes duration and strength of locomotor activity, thereby ensuring a reliable coupling between locomotion and tentacle motion. The strict phase coupling between the trigeminal and spinal motor activity, present in many cases, suggests that the respective corollary discharge is causally related to the ongoing locomotor output and derives at least in part from the spinal central pattern generator; however, additional contributions from midbrain and/or hindbrain locomotor centers are likely. The swimming-related retraction might protect the touch-receptive Merkel cells on the tentacle from sensory over-stimulation and damage and/or reduce the hydrodynamic drag. The intrinsic nature of the coupling of tentacle retraction to locomotion is an excellent example of a context-dependent, direct link between otherwise discrete motor behaviors.

KEY WORDS: Corollary discharge, Efference copy, Spinal locomotion, Trigeminal nerve, *Xenopus laevis*

INTRODUCTION

During rhythmic locomotion, a number of otherwise independent movements are influenced by the rhythm of the motor commands. In vertebrates, such coupling is observed for respiration (Bramble and Carrier, 1983), eye movements (Lambert et al., 2012), tail motion (Wada et al., 1993) or phase-locked arm and trunk motor adjustments (Earhart, 2013). While some coupled motor behaviors such as tail motion, e.g. in dogs, improve locomotor performance (Wada et al., 1993), others improve sensory acquisition and processing as observed for retinal image stabilization (Chagnaud et al., 2012a; Lambert et al., 2012). One possibility for coupling otherwise unrelated motor behaviors to locomotion is via corollary discharge that derives from spinal or supraspinal locomotor centers. These signals allow fast and reliable phase locking of the respective motor behavior to the locomotor rhythm (Chagnaud et al., 2012a). Corollary discharges are independent of locomotor style and occur during rhythmic limb-based locomotion in terrestrial vertebrates as well as during body/tail-based swimming in aquatic vertebrates (Chagnaud et al., 2012a). Traditionally, motor corollary discharges have been described as mechanisms to differentiate between environmental and self-generated sensory inputs (Crapse and Sommer, 2008; Cullen, 2004, 2011; von Holst and Mittelstaedt, 1950; Poulet and Hedwig, 2007; Sommer and Wurtz, 2008). In contrast, the impact of motor corollary discharges on motor systems and the indirect effects on the sensory encoding have been less well investigated. Some studies have shown that corollary discharge can cause reflex inhibition during swimming in *Xenopus* embryos (Sillar and Roberts, 1988), suppress withdrawal responses in gastropods during feeding behavior (Davis et al., 1974; Kovac and Davis, 1980) and drive compensatory eye movements during swimming in larval *Xenopus* (Lambert et al., 2012). However, attempts to decipher the mechanisms underlying the effects of corollary discharge on motor behaviors are often constrained by the complexity of the central nervous system and the limb-based locomotion. In contrast, tail-based swimming in amphibians (Wassersug and Hoff, 1985) and fishes represents a simple, stereotyped locomotor pattern with correspondingly simpler spatio-temporal profiles of corollary discharges.

A number of aquatic anamniotes possess various numbers of mobile appendages on the head that contain several types of sensors (see Fox, 1999). One example is the pair of rostrally protruding tentacles in *Xenopus laevis* Daudin tadpoles that are equipped with Merkel cells (Ovalle, 1979). The mechanosensory nature of these cells (Maricich et al., 2009) suggests an important role for touch discrimination and surface structure recognition. Because of the location of the tentacles on the head and the tadpole's undulatory swimming style with prominent horizontal head oscillations, the tactile function of these appendages might be impaired during locomotor behavior. Given the previously reported spinal efference copy-driven compensatory eye motion in *Xenopus* tadpoles during swimming (Combes et al., 2008; Lambert et al., 2012), a protection of the sensor and a potential improvement of propulsive efficacy could be achieved by a similar spinal locomotor corollary discharge that causes a tentacle retraction.

Here, we provide direct evidence that locomotor corollary discharges during rhythmic swimming in *X. laevis* tadpoles initiate a bilateral tentacle retraction. Fluorescent tract tracing, Ca^{2+} imaging, electrophysiological recordings and video analysis of tentacle motor behavior during fictive locomotion in semi-intact *in vitro* preparations outline the underlying trigeminal motoneuronal populations, their firing pattern and their link to ongoing locomotor commands. The observed coupling of tentacle retraction to swimming is an excellent example of intrinsic control of a particular motor behavior that is otherwise unrelated to locomotion.

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Received 16 February 2015; Accepted 30 March 2015

RESEARCH ARTICLE The Journal of Experimental Biology (2015) 218, 1748-1758 doi:10.1242/jeb.120824

RESULTS

Locomotion-coupled tentacle motion

Xenopus laevis tadpoles are equipped with a bilateral, mobile pair of tentacles (Cannone and Kelly, 1977; Ovalle, 1979) that are attached to the upper jaw at the lateral aspect of the mouth. At rest, these appendages extend rostro-laterally (Fig. 1Ai,B) at an angle of 19±10 deg (mean±s.d., 61 tentacles) relative to the longitudinal body axis. During fictive locomotion, indicated by rhythmic bursts in spinal ventral roots (VRs) in isolated tadpole preparations (Combes et al., 2004; see below for details), video recordings revealed that the tentacles were concurrently retracted (Fig. 1Aii). The maximal retraction angle during swimming was 115±24 deg (61 tentacles, n=41 swimming episodes in N=7 preparations; Fig. 1B). During locomotor activity, the tentacles essentially remained in a lateral position interspersed by variably timed and sized oscillations (Fig. 1C). As the locomotor activity ceased, the tentacles protracted towards the initial resting position (Fig. 1Aiii, Aiv, Ci).

Simultaneous video recordings of the tentacles on both sides (n=20 episodes in N=4 preparations) yielded very similar motion dynamics and trajectories of both tentacles during a given locomotor episode (see red and blue traces in Fig. 1Ci). The close coupling of onset, duration and motion pattern of the left and right tentacle was verified by correlating the respective motion trajectories (Fig. 1Cii). The distribution of the correlation coefficients (median: 0.81; Fig. 1D) was significantly different from zero (P<0.0001; Wilcoxon signed-rank test) indicating that both tentacles move symmetrically. This close match of the bilateral motor activity was exploited in subsequent experiments by combined recordings of the motion pattern of one tentacle and the neuronal commands of the trigeminal motor nerve that innervates the tentacle on the other side. Moreover, the presence of tentacle movements during locomotion in completely isolated and immobile *Xenopus* tadpole preparations and thus in the absence of tail motion-related sensory signals suggests that the motor commands derive from a locomotor corollary discharge that activates the tentacle motor system.

Anatomical organization of the tentacle motor system

Tentacle movements in *Xenopus* tadpoles are produced by a single muscle (m. levator mandibulae pars lateralis) that retracts the tentacle (Nieuwkoop and Faber, 1994); in contrast, protraction of the tentacle occurs in the absence of a respective muscle but likely involves the mechanics of a cartilaginous spring (Ovalle et al., 1998). The tentacle muscle, responsible for the retraction, has a joint proximal origin with the jaw closing muscles (m. levator mandibulae pars intermedius and medialis) but remains otherwise separate (dashed line in Fig. 2B), and attaches distally at the cartilaginous base of the tentacle (Fig. 2A,B). The exclusive control of tentacle movements by the lateral portion of the m. levator mandibulae was demonstrated by a set of differential muscle lesions. A specific transection (three preparations) of the m. levator mandibulae pars lateralis, which left the other two portions of the m. levator mandibulae intact, abolished all tentacle movements. In contrast, a reciprocal surgical intervention (three preparations), that preserved the lateral portion, left tentacle movements unaffected. The sensory-motor innervation of the tentacle and its muscle is formed by the trigeminal ophthalmic (rOP) and mandibular branches (rMA) as illustrated by the pattern of labeled peripheral nerves following application of biocytin to the trigeminal nerve root close to the hindbrain (Fig. 2A). The motor innervation of the tentacle muscle, however, originates exclusively from the smaller branch (tentacle nerve, TN) after ramification of the rMA into two branches at the level of the m. levator mandibulae (red asterisk in Fig. 2B).

Apart from innervating the tentacle muscle, the TN branch also innervates the jaw closing muscle (Fig. 2A,B). This branch exclusively contains motor axons as indicated by the absence of sensory hindbrain projections following tracer application to this particular nerve (left side in Fig. 2C). This differs from the observed pattern after labeling of the second rMA branch (blue arrowhead in Fig. 2B) or the trigeminal nerve root, where

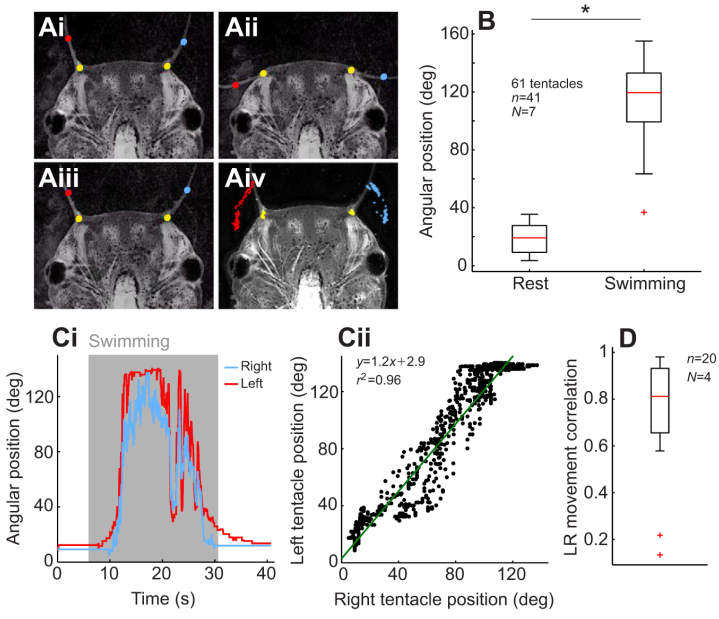

Fig. 1. Bilaterally coordinated tentacle movements during fictive swimming in an isolated *in vitro* *Xenopus laevis* tadpole preparation. (A) Representative frames from a video recording before (Ai), during (Aii) and after (Aiii) an episode of fictive swimming; positions of the left and right tentacle and their proximal insertions (yellow) are superimposed on an image overlay (Aiv) visualizing the trajectories during the entire locomotor event. (B) Boxplots of angular tentacle positions at rest and at maximal eccentricity (relative to the longitudinal body axis) during fictive swimming (61 tracked tentacles, obtained from n=41 locomotor episodes in N=7 preparations). (Ci) Angular trajectory of the left and right tentacle during an episode of fictive swimming (gray area); (Cii) scatter plot and linear regression correlating the angular positions of the two tentacles during the locomotor episode shown in Ci. (D) Boxplot of correlation coefficients between angular positions of the left and right (LR) tentacles during n=20 swimming episodes in N=4 preparations with a median coefficient of 0.81 (P<0.0001; Wilcoxon signed-rank test).

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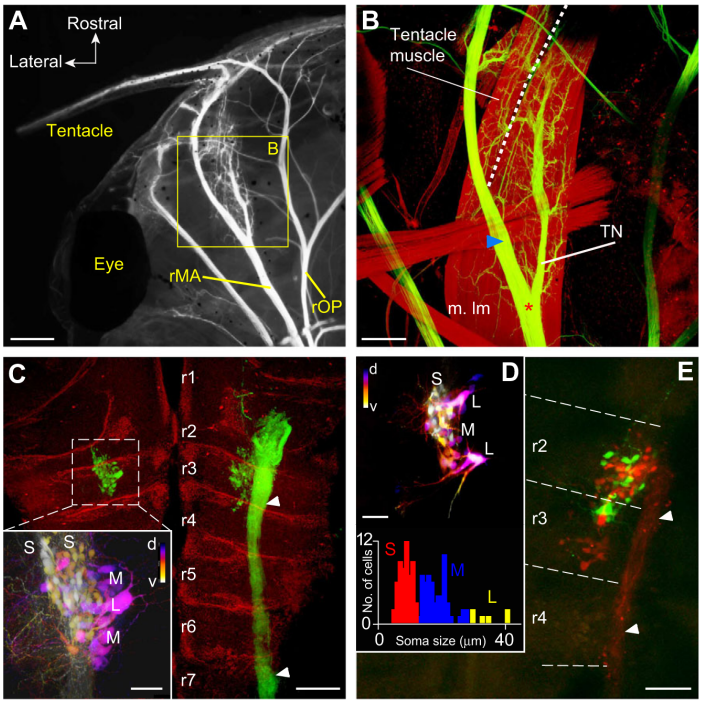

Fig. 2. Anatomical organization of the tentacle motor system. (A) Wide-field fluorescence image, depicting the left tentacle and its biocytin/streptavidin-Cy2-labeled (in white) neuronal innervation by the ophthalmic (rOP) and mandibular branch (rMA) of the trigeminal nerve. (B) Confocal reconstruction of the area outlined in A, illustrating the tentacle muscle – lateral portion (dashed line) of the m. levator mandibulae (m. lm) – and the tentacle nerve (TN) that branches off (red asterisk) from the main rMA (blue arrowhead); confocal scanning at 488 and 633 nm visualized biocytin/streptavidin-Cy2-labeled trigeminal nerve branches (green) and muscle tissue (red). (C–E) Confocal reconstruction of trigeminal motoneurons and afferent projections in the hindbrain (white arrowheads in C, E), labeled with biocytin/streptavidin-Cy2 or Alexa Fluor dextran 488/546 from the TN (left side in C, D; green neurons in E), the trigeminal nerve root (right side in C) or the main rMA (red neurons in E) distal to the TN branching (blue arrowhead in B); rhombomere (r1–7) boundaries (C) were visualized with 633 nm illumination; tentacle motoneurons, color coded according to their position along the *z*-axis (inset in C, D), subdivide into dorso-medially located large (L) and medium-sized (M) elongated cells and ventro-laterally located small, round cells (S); the dorso-ventral extension of the *z*-stack is 74 µm in the inset in C and 128 µm in D. The histogram in the lower part of D displays the distribution of soma diameters across preparations. Color code: d, dorsal; v, ventral. Scale bars are 0.5 mm in A, 0.2 mm in B, C and E, and 50 µm in C inset and D. Arrows in A indicating the rostral and lateral direction apply to all other panels.

additional dense afferent terminations were observed in the dorsal hindbrain throughout r1–r7 (arrowheads on the right side in Fig. 2C,E), indicating the presence of sensory fibers in the respective nerves.

The hindbrain location of motoneurons innervating the tentacle muscle was compared with the entire population of trigeminal motoneurons following application of biocytin to the TN at its entrance into the muscle or to the trigeminal nerve close to the hindbrain (cell groups on the left and right side, respectively, in Fig. 2C). Tracer application to the TN consistently labeled a group of motoneurons in hindbrain segments r2 and r3 (left side in Fig. 2C–E). This bi-segmental location coincides with that of the entire population of trigeminal motoneurons, labeled from the trigeminal nerve root (right side in Fig. 2C). The number of retrogradely labeled tentacle motoneurons was variable and ranged from 9 to 87 neurons in different experiments (median: 27 cells, N=10 preparations). These motoneurons only innervated the m. levator mandibulae because labeling of the TN and the second rMA branch (blue arrowhead in Fig. 2B) in different colors (Alexa Fluor dextran 488 and 546; N=4 preparations) did not yield double-labeled neurons (see absence of yellow cells in r2 and r3 in Fig. 2E). However, tentacle motoneurons were intermingled with other rMA motoneurons (green and red cells in r2 and r3 in Fig. 2E) within the trigeminal nucleus.

Tentacle motoneurons form a heterogeneous population with respect to soma size and shape (inset in Fig. 2C,D). A histogram of soma diameters (120 cells in N=6 preparations) revealed that cell size extends over a large range that suggests the presence of up to three groups of neurons with a tendency for a size-related dorso-ventral separation (see Fig. 2C,D inset for *z*-axis color-coded confocal reconstruction). Neurons were categorized as either small (<12 µm) and mostly round (group 1: S) or medium sized (≥12 and <30 µm) with oval somata (group 2: M) with the consistent exception of one to two very large (≥30 µm) neurons with elongated cell bodies (group 3: L), located at the most dorso-medial aspect of the labeled cell group (L in Fig. 2C,D inset). Medium-sized neurons were located at more medial and dorsal positions relative to the smaller cells (Fig. 2C,D inset). The mean size of the neurons was 8.3±1.9 µm (56 cells) for small neurons, 18.1±3.8 µm (58 cells) for medium neurons and 34.6±4.4 µm (six cells) for large neurons. Accordingly, tentacle motoneurons form a morphologically diverse and dispersed cell group within the trigeminal motor nucleus in r2 and r3 with a size-related dorso-ventral arrangement.

Activity of tentacle motoneurons during locomotion

The morphological diversity of tentacle motoneurons prompted us to test whether all or only a particular subpopulation of motoneurons become active during swimming and cause the observed tentacle retraction. Neuronal activity was measured as Ca^{2+} transients in the entire tentacle motor nucleus or in individual motoneurons, following unilateral or bilateral application of Calcium Green-1 dextran to the peripheral motor target(s) and retrograde transport to the cell bodies (Fig. 3Ai inset, Bii, Cii). During episodes of fictive locomotor activity, visible as rhythmic bursting in the spinal VR (green trace in Fig. 3Ai), the population of tentacle motoneurons showed fluorescence changes that were timed to the VR bursting (Fig. 3Ai). Simultaneous Ca^{2+} imaging of the bilateral tentacle motor nuclei (red and blue outline in Fig. 3Ai inset) yielded almost identical dynamics of the transients (red and blue traces in Fig. 3Ai), matching the symmetric motion profiles of the two appendages (Fig. 1Ci,D). The duration of these population responses (n=15 swimming episodes in N=8 preparations), measured as half-width of the Ca^{2+} transients (Fig. 3Ai), was highly correlated with the

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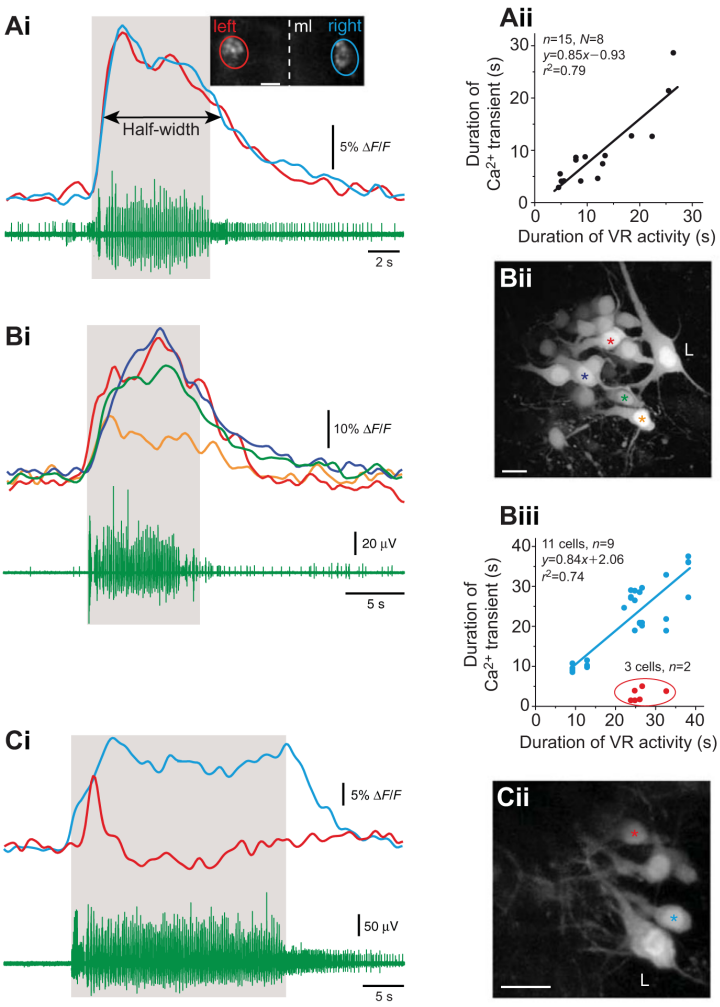

Fig. 3. Calcium dynamics in tentacle motoneurons during fictive locomotion. (Ai) Population Ca^{2+} transients (red and blue traces) of the tentacle motor nuclei on both sides (red and blue encircled areas in inset) during an episode of fictive swimming (gray area), indicated by the spinal ventral root (VR) burst discharge (green trace); motoneurons were retrogradely labeled with the Ca^{2+} sensor (Calcium Green-1 dextran) from the target muscle. (Aii) Scatter plot and linear regression of population Ca^{2+} response duration (half-width) as a function of the duration of rhythmic VR activity during n=15 swimming episodes in N=8 preparations (r^2=0.79, P<0.0001, Wilcoxon signed-rank test). (B,C) Ca^{2+} transients (color-coded traces in Bi, Ci) of individual tentacle motoneurons (color-matched asterisks in Bii, Cii) during episodes of fictive swimming (gray areas in Bi, Ci). (Biii) Scatter plot and linear regression of the Ca^{2+} response half-width in single neurons (blue dots) as a function of the duration of rhythmic VR activity (r^2=0.74, P<0.0001, Wilcoxon signed-rank test); note the small group of motoneurons (red dots) with highly phasic Ca^{2+} transients (red trace in Ci) during fictive swimming (no significant correlation, P>0.05). Data in B and C are from 14 cells during n=9 swimming episodes in N=3 preparations (31 data points). L, large neurons (see Fig. 2D); ml, midline. Scale bars are 100 µm in inset of Ai, 20 µm in Bii and Cii.

duration of the fictive swimming episodes (Fig. 3Aii). Thus, the duration of Ca^{2+} responses in the tentacle motor nuclei closely matches the duration of a swimming episode.

In a separate set of experiments, we determined the fraction and morphology of the activated tentacle motoneurons by recording Ca^{2+} responses of individual motoneurons (34 cells; n=9 swimming episodes in N=3 preparations; Fig. 3Bii,Cii). Ca^{2+} transients were encountered in 14 out of 34 retrogradely identified motoneurons during fictive swimming episodes (Fig. 3Bi,Ci). Responses were observed only in the population of medium-sized motoneurons with oval cell bodies (16.1±1.6 µm, 14 cells), whereas neither the subgroup of small motoneurons nor the one to two very large motoneurons displayed any locomotion-related responses (Fig. 3B,C). Based on the dynamics of the Ca^{2+} responses, the 14 responsive neurons could be separated into a majority of cells (11 cells; color-coded traces in Fig. 3Bi and blue trace in Fig. 3Ci) with a half-width of the transients that significantly correlated with the duration of the corresponding swimming episode (blue dots in Fig. 3Biii). In contrast, a few motoneurons (two swimming episodes in three neurons) exhibited Ca^{2+} responses that were highly transient and only present at the beginning of a swimming episode (red trace in Fig. 3Ci; red dots in Fig. 3Biii). However, the morphology of these neurons was indistinguishable and the size not significantly different from the size of those with the predominant response pattern. Thus, motoneurons responsible for tentacle motion during tadpole swimming appear to form a morphologically homogeneous group with Ca^{2+} response profiles that suggest the presence of either transient or sustained activity during locomotor episodes.

Dynamics of tentacle motor commands

To reveal the discharge profile of the motor commands that provoke tentacle movements during locomotor CPG activity, we made simultaneous recordings of a VR on one or both sides of the spinal cord, the TN on one side and the motion of the tentacle on the other side (Fig. 4A). During fictive swimming, indicated by an episode of burst activity in VRs (green and gray traces in Fig. 4B), the otherwise silent TN became active (black trace and firing rate

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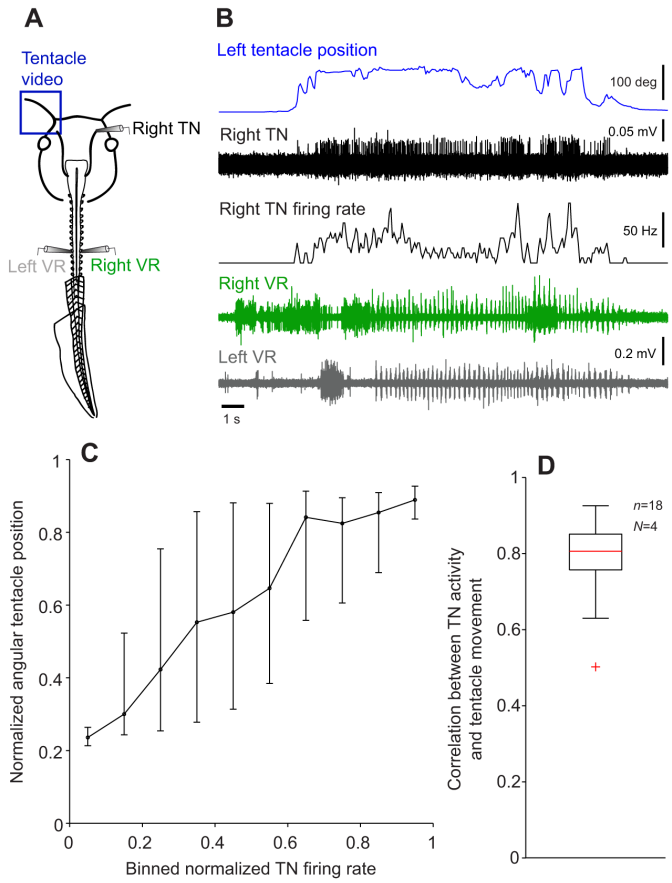

Fig. 4. Correlation between TN motoneuronal activity and tentacle movement during fictive locomotion. (A) Sketch depicting simultaneous video imaging of the left tentacle and electrophysiological recording of the right TN and bilateral spinal VRs in a semi-intact tadpole preparation. (B) Tentacle motion (blue trace), TN spike activity and instantaneous firing rate (black traces) and bilateral VR discharge (green and gray traces) during fictive swimming. (C) Median and 25th and 75th percentiles of normalized tentacle position plotted against normalized and binned TN firing rate of n=18 swimming episodes in N=4 preparations. (D) Boxplot illustrating the distribution of the correlation coefficients between the firing rate of the TN on one side and the movement of the tentacle on the other side (red cross indicates outliers) for the swimming episodes in C with a median of 0.81 (the distribution is significantly different from zero, P<0.001; Wilcoxon signed-rank test).

in Fig. 4B). In addition, the intact tentacle contralateral to the recorded TN was simultaneously retracted (blue trace in Fig. 4B). The similarity of tentacle motion and tentacle motor profiles (blue and black traces in Fig. 4B) was quantified by correlating the normalized position of the tentacle with the normalized firing rate of the contralateral TN (Fig. 4C). Normalized tentacle motion and contralateral TN firing rates were correlated with a coefficient ranging between 0.50 and 0.93 (median: 0.81; n=18 swim episodes in N=4 preparations; Fig. 4D). The distribution of correlation coefficients was significantly different from zero (P<0.001; Wilcoxon signed-rank test). Despite the variability in the movements of both tentacles (Fig. 1Ci), the distribution of correlation coefficients indicates that the firing pattern of the TN on one side faithfully predicts the motion profile of the tentacle on the other side.

Coupling properties of locomotor and tentacle motor activity

Locomotor influence on tentacle motion was studied by analyzing the coupling pattern between spinal and tentacle motor activity. Tadpole swimming is a fluctuating motor behavior in terms of duration and strength/speed, which is reflected during fictive locomotion by variable episode durations, burst frequencies and amplitudes (Combes et al., 2004). By comparing spinal VR and TN activity, we identified those parameters of the locomotor commands that are transmitted to the tentacle motor system. The most obvious of these parameters was the duration of the fictive swimming episode: the discharge in spinal VRs and in the TN closely matched in time (compare black with green traces in Fig. 5A), as indicated by the significant linear regression with a slope close to unity (Fig. 5B; r^2=0.94, P<0.0001, Wilcoxon signed-rank test, n=57 swim episodes in N=10 preparations). This agrees with the behavioral observation that the tentacles are retracted during the entire swimming event.

Alterations in swimming strength *in vivo* essentially derive from changes in the magnitude and frequency of tail excursions that in turn are represented *in vitro* by variations in the cycle frequency of the VR bursts and in the intra-burst firing rate (Combes et al., 2004). A spontaneous increase in spinal VR intra-burst discharge, represented by a larger integral of the firing rate (green traces in Fig. 5C) was accompanied by a similar increase in retraction angle of the tentacle (blue trace in Fig. 5C). With increasing locomotor strength from low to medium to high swimming amplitudes (Fig. 5D), the tentacle reached progressively more eccentric positions. In fact, the tentacle positions for the three levels of swimming strength were significantly different from each other (P<0.0001, Kruskal–Wallis test and *post hoc* comparisons; n=4 swimming episodes in N=3 preparations). The variation of position with swimming strength prompted us to compare the swimming

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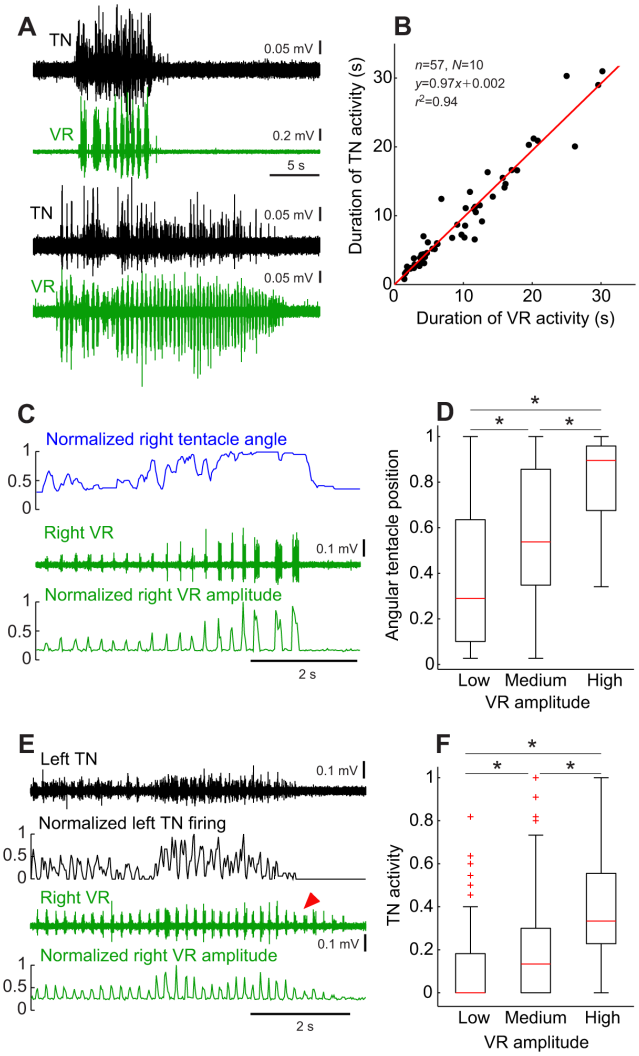

Fig. 5. Tentacle motor commands contain information about fictive swimming duration and amplitude. (A) TN discharge (black traces) and VR burst activity (green traces) during two fictive swimming episodes of different length in the same preparation. (B) Scatterplot and linear regression of TN discharge duration as a function of the duration of VR activity during n=57 swimming episodes in N=10 preparations (r^2=0.94). (C) Angular trajectory of the tentacle (normalized to maximal excursion, blue trace), ipsilateral VR activity with increasing burst amplitude (upper green trace) and integrated burst amplitudes (normalized to maximal value, lower green trace) during an episode of fictive swimming. (D) Boxplot of tentacle angular position as a function of VR burst amplitude; normalized VR burst amplitudes were binned into three levels (low, medium and high); normalized tentacle angular positions, obtained from n=4 swimming episodes in N=3 preparations, were significantly different between the three groups (P<0.0001; Kruskal–Wallis test and *post hoc* comparisons). (E) TN discharge (upper black trace), contralateral VR burst activity (upper green trace) and normalized TN and VR firing rate amplitudes (to maximal magnitude; lower black and green traces, respectively) during fictive swimming with modulated VR burst amplitudes. (F) Boxplot (red crosses indicate outliers) of TN firing rate as a function of VR burst amplitude; normalized VR burst amplitudes were binned into three levels (low, medium and high); normalized TN firing rate amplitudes – obtained from n=4 swimming episodes in N=3 preparations – were significantly different between the three groups (P<0.0001, Kruskal–Wallis test and *post hoc* comparisons).

strength (green traces in Fig. 5E) during a given locomotor episode with the rate of TN firing (black traces in Fig. 5E). Spontaneous alterations of VR burst amplitude caused a comparable modulation of the TN firing rates, with the latter discharge ceasing as soon as the VR bursting decreased below a particular level (red arrowhead in Fig. 5E). Pooling the data from four different swimming episodes in three preparations where the VR burst discharge frequency displayed a spontaneous modulation showed that the TN activities at the three levels of swimming strength were also significantly different from each other (Fig. 5F; P<0.0001, Kruskal–Wallis test with *post hoc* comparisons), with a more pronounced TN activity during stronger swimming.

While the previously described corollary discharge in extraocular motoneurons during rhythmic locomotion in *Xenopus* tadpoles is strictly phase coupled to the VR bursting in a 1:1 fashion (Lambert et al., 2012), the dynamics of tentacle motor commands appears to be more variable and temporally more complex (Fig. 6). To assess the coupling of TN spiking during episodes of fictive swimming as a function of the timing of VR bursts, we generated VR burst-triggered cumulative spike time histograms (see Materials and methods). Based on entropy statistics (Kajikawa and Hackett, 2005), recordings from 18 out of 31 swimming episodes in N=7 preparations (58%) showed significant phase coupling between spinal VR and TN discharge (Fig. 6A,B,D), while the TN discharge during 13 swim cycles displayed no coupling to the VR burst rhythm (Fig. 6C). In the group of phase-coupled recordings, a mono-phasic coupling (four out of 18; Fig. 6A) could be clearly distinguished from a bi-phasic pattern (five out of 18; arrowhead in Fig. 6Bii inset), while the remainder (nine out of 18) exhibited a phase coupling that could not be unambiguously distinguished as one or the other. The different coupling patterns during individual swimming bouts were summarized as circular plots, emphasizing the variability among the different coupling categories (Fig. 6Aiii–Ciii). Functionally, the mono- or bi-phasic timing of TN bursts (Fig. 6Aiii, Biii) suggests that

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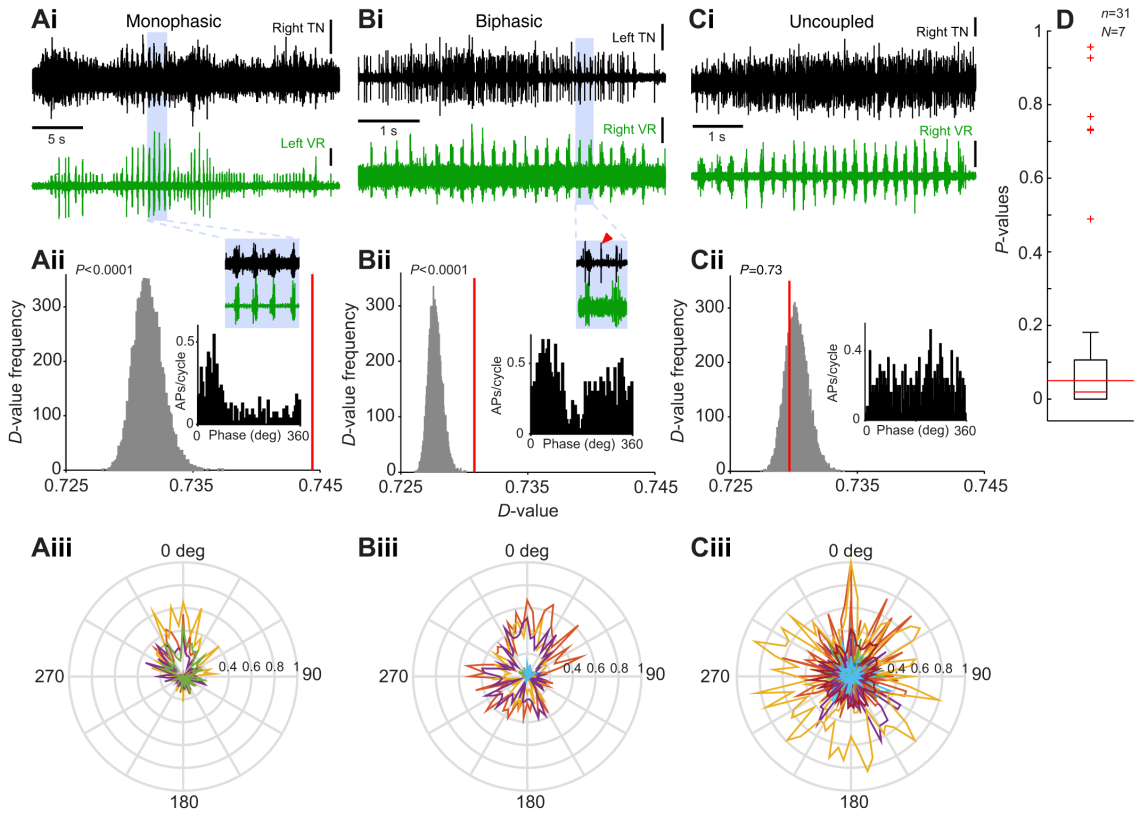

Fig. 6. Phase coupling between TN and VR activity during fictive locomotion. (A–C) TN discharge (black traces) and contralateral (Ai, Bi) or ipsilateral (Ci) VR burst activity (green traces); VR–TN coupling subdivides into mono-phasic (Ai), bi-phasic (red arrowhead in Bi) and uncoupled patterns (Ci) as indicated by the insets on an extended time scale (blue background); distribution of *D*-values (Aii, Bii, Cii) from re-shuffled inter-spike intervals (see Materials and methods) in the TN recording from the three examples (Ai, Bi, Ci) assessed the significance of phase coupling; the *D*-value from the original trace is indicated by the red vertical line; insets in Aii, Bii and Cii show the spike histograms of the TN discharge relative to the swimming phase. Circular plots in Aiii, Biii and Ciii summarize the distribution of TN spikes across VR cycles for individual swimming bouts (color coded). (D) Boxplot of the *P*-value distribution obtained from *n*=31 swimming episodes in *N*=7 preparations to assess the significance of phase coupling based on the *D*-value. Scale bars: Ai TN, 0.05 mV; VR, 0.2 mV, Bi TN, 0.2 mV; VR, 0.05 mV, Ci TN, 0.05 mV; VR, 0.1 mV.

the inputs to trigeminal motoneurons originate from one or both sides of the spinal cord during a particular recording. This might also explain the difficulty of assigning a particular phase relation to the coupling pattern in most cases (mono- versus bi-phasic), assuming a continuum between unilateral and bilateral locomotor corollary drive to tentacle motoneurons.

DISCUSSION

During fictive swimming in *Xenopus* tadpoles, a corollary discharge informs the tentacle motor system about ongoing locomotor activity. This intrinsic locomotor signal activates a discrete set of trigeminal motoneurons on each side of the rostral hindbrain that causes a symmetrical retraction of the appendages. The motor command encodes both duration and magnitude of the locomotor activity, thereby ensuring a reliable coupling between propulsive locomotion and tentacle motion. Given the phase coupling between trigeminal nerve discharge and spinal motor rhythm, the locomotor corollary discharge derives at least in part from the spinal central pattern generator (CPG) circuitry.

Function of tentacles

Xenopus laevis tadpoles possess a pair of mobile appendages between developmental stage 47 and 61 (Nieuwkoop and Faber, 1994), which contain Merkel cells and thus were suggested to play a role in touch reception (Ovalle, 1979; Ovalle et al., 1998). These tentacles are usually protruded forward but can be actively retracted into a lateral position (Fig. 1A). The use of appendages for mechanoreceptive exploration of the environment in these animals is useful for near-field orientation and navigation in the mostly murky aquatic environment in which these animals naturally live (Nieuwkoop and Faber, 1994). A similar mechanoreceptive role in mammals is performed by mechanoreceptive facial hairs – whiskers. Rodents use whisker movements for tactile exploration of the environment, for instance while walking along the wall of a cage (Hartmann, 2011). Similarly, tadpoles drift slowly with their tentacles touching the walls and floor of the tank (S.H., R.B., H.S. and B.P.C., unpublished observation), potentially collecting tactile information. However, several differences between the two structures exist: tentacles are formed by a single protrusion located on each side of the head, while whiskers are usually arranged in

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groups (Berg and Kleinfeld, 2003). Moreover, whisker movements in rodents are controlled by multiple muscles (Berg and Kleinfeld, 2003), while tentacle motion in *Xenopus* tadpoles is exerted by a single muscle and a spring-like antagonistic mechanism (Ovalle et al., 1998). For tactile exploration, whiskers are actively moved ('whisking'), while tentacles are kept in an extended position. However, despite these morphological differences, amphibian tentacles appear to be similar, yet simpler versions of touch-receptive mammalian appendages.

Potential function of tentacle retraction

The presence of Merkel cells at a relatively high density in *Xenopus* tentacles (Ovalle, 1979) indicates a particular sensitivity of these structures to touch. Because of the hydrodynamic drag between the appendages and water, permanently extended tentacles could rhythmically stimulate these touch receptors during swimming-related head undulations. A tonic retraction of the tentacles during locomotor episodes might considerably reduce excessive stimulation of the sensory cells, in particular during strong and fast swimming. Under this assumption, locomotor corollary discharge-mediated tentacle retraction would minimize self-generated stimulation of the Merkel cells during swimming by reducing the water flow/pressure impinging on the tentacles. In a number of other sensory systems, corollary discharge similarly reduces or even suppresses self-generated sensory inputs (for review, see Crapse and Sommer, 2008); this also complies with the classical role of motor efference copies or corollary activity (von Holst and Mittelstaedt, 1950; Sperry, 1950). As a side effect, tentacle retraction during locomotion reduces the likelihood of mechanical damage to the tentacles that can reach a maximal length of 1 cm (~20% of larval body length).

In addition to this putative protective function, tentacle retraction might also improve the hydrodynamics of the tadpole. Appendage retraction streamlines the body shape, thereby facilitating energetically more efficient swimming (Crespi et al., 2013; Liu et al., 1997). This is particularly important during strong swimming, when protruded tentacles would cause a considerable drag and impair the sinusoidal motion of the head during swimming, specifically at those larval stages where these appendages are at their maximal length. In fact, a similar streamlining of the body shape is observed during tail-based swimming in other amphibians such as salamanders, where both forelimbs and hindlimbs are aligned to the body (Delvolvé et al., 1997) or in axolotl, where the external gills are retracted (D'Août and Aerts, 1997). Thus, locomotor-coupled head appendage or limb retractions in amphibians represent distinct behavioral reactions that improve the hydrodynamic signature and, in the case of retracted limbs during tail-based swimming, considerably enhance locomotor performance (Crespi et al., 2013). Such a strategy is not restricted to amphibians but occurs also during swimming in alligators (Manter, 1940; Fish, 1984) and 'terrestrial swimming' in sandfish lizards (Maladen et al., 2009) where the tail-based propulsion is accompanied by similar limb adductions as in salamanders (Delvolvé et al., 1997).

Origin of corollary discharge in trigeminal/tentacle motoneurons

The absence of propulsive movements and movement-related sensory feedback in semi-intact *Xenopus* preparations (Straka and Simmers, 2012) demonstrates that the coupling between locomotion and tentacle motion relies exclusively on intrinsic signals, i.e. corollary discharge from locomotor areas. Theoretically, these signals could derive from midbrain locomotor centers (Cabelguen et al., 2003; Saitoh et al., 2007) or from CPGs in the rostral spinal cord or from a combination of the two.

A spinal contribution of the corollary activity in tentacle motoneurons is supported by the presence of strict phase coupling of the TN burst discharge with the burst rhythm of the spinal VR on one (mono-phasic) or on both sides (bi-phasic; Fig. 6A,B) along with the highly correlated amplitudes of spinal VR and TN firing or tentacle motion. The absence of a clear VR–TN burst coupling in a number of experiments (Fig. 6C) might reflect the consequence of signal integration in a particular motoneuronal population that only becomes apparent in some experiments depending on the number and variety of recorded TN axons. Alternatively, or in addition, the ascending spinal locomotor corollary discharge in tentacle motoneurons, unlike in extraocular motoneurons (Lambert et al., 2012) might be supplemented by tonic signals from locomotor centers in the midbrain and/or hindbrain. In fact, midbrain neurons in the salamander *Notophthalmus viridescens*, for instance, display tonic activity during locomotion (Cabelguen et al., 2003), similar to that seen in the TN nerve during locomotor episodes. Even though the relative contributions of midbrain locomotor regions and spinal CPGs to the tentacle motor corollary discharge remain unclear, we hypothesize that both neuronal structures contribute to the trigeminal activation during locomotor activity.

Evolutionary origin of spinal–hindbrain coupling

While locomotion-related tentacle retraction potentially serves several distinct, not mutually exclusive purposes (see above), the coupling could be a vestige of the rostro-caudally distributed activity in the spinal cord-like nervous system of vertebrate ancestors during undulatory swimming (Fetcho, 1992; Wada, 1998). However, given the rather specialized structure and function of these rostral appendages, this notion appears too simplistic. The inherent linkage between the two motor systems rather suggests a particular functional role of tentacle retraction during swimming, which is not necessarily exclusive or in opposition with a pre-existing vestigial signaling pathway. Accordingly, ascending locomotor corollary discharges from the spinal CPG circuitry might represent a widely distributed signaling component within the brain during rhythmic locomotion and might influence numerous sensory and/or motor systems. Among the hindbrain motor systems currently known to receive spinal inputs are the extraocular system (Combes et al., 2008; Lambert et al., 2012; von Uckermann et al., 2013) and the trigeminal motor component (this study). Evidence for an even more widespread function arises from studies on spiny dogfish (Russell and Roberts, 1974) and larval *Xenopus* (Chagnaud et al., 2012b), in which connections from the spinal cord locomotor CPG to mechanosensory efferent neurons were demonstrated. Further hindbrain targets might include hindbrain nuclei in axolotl that cause a retraction of the gills during swimming (D'Août and Aerts, 1997).

Based on the various examples, we hypothesize that appendage retraction (e.g. limbs, tentacles, gills) during undulatory swimming is generally caused by locomotor corollary activity, which allows a fast and reliable coupling of independent motor behaviors with locomotion. Such a functional role expands the traditional concept of motor efference copies/corollary discharge in simply differentiating between external and self-generated sensory signals (Cullen, 2004, 2011). Accordingly, corollary discharge during locomotor activity appears to influence a large number of both sensory and motor systems throughout the brain, including the

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trigeminal system, potentially optimizing locomotor performance and sensory perception (Chagnaud et al., 2012a). Depending on the type of motor behavior (rhythmic, task oriented), the effect of corollary activity might vary considerably (Crapse and Sommer, 2008; Cullen, 2014; King, 2013). Nonetheless, intrinsic coupling offers a convenient substrate for a context-dependent, direct linkage between otherwise discrete motor behaviors.

MATERIALS AND METHODS

Experiments were performed on semi-intact *in vitro* preparations of 33 larval *Xenopus* at stages 51–55 (Nieuwkoop and Faber, 1994) in compliance with the 'Principles of Animal Care' publication by the National Institutes of Health and the German law for animal protection (Tierschutzgesetz). Permission for the experiments was granted by the Regierung von Oberbayern (55.2-1-54-2531.3-18-10). All animals were obtained from the in-house breeding facility at the Biocenter-Martinsried of the LMU Munich.

Preparations

In all experiments, animals were first anesthetized in 0.02% 3-aminobenzoic acid ethyl ester (MS-222; Sigma-Aldrich Pharma Ltd, UK) in ice-cold frog Ringer solution (composition in mmol l^{-1}: NaCl, 75; KCl, 2; $CaCl_2$, 2; $MgCl_2$, 0.5; $NaHCO_3$, 25; glucose, 11: pH 7.4). The ventral part of the head/body, including the lower jaw and visceral organs, was carefully removed, leaving the tail attached to the head and the sensory-motor innervation of the tentacles intact. The skin covering the dorsal portion of the head was removed, the soft skull tissue opened and the forebrain disconnected. Preparations were transferred to a Petri dish (volume 5 ml) and fixed dorsal-side up to the Sylgard floor with insect pins. In some cases the spinal cord was exposed up to segment 20 and the VRs were transected bilaterally. The remaining, caudal part of the tail was firmly secured on both sides to the Sylgard floor with insect pins at the level of segments 21–25, with the remainder of the tail left free to perform undulatory swimming movements. After repeated rinsing in fresh Ringer solution, preparations were continuously superfused with oxygenated Ringer solution at a rate of 1.3–2.1 ml min^{-1}. The temperature of the bath solution was maintained at 17±0.5°C. Recordings lasted up to 5 h.

Tentacle motor behavior during spinal VR activity (i.e. fictive swimming, hereafter referred to as swimming) in these isolated preparations was quantified by video analyses of tentacle motion and/or recording the respective motor nerve discharge. For monitoring tentacle movements during fictive swimming, one or both tentacle(s) remained attached and innervated by the respective trigeminal nerve branch. Motor commands for tentacle movements were captured after further unilateral or bilateral isolation of the trigeminal motor nerve branch that innervates the tentacle muscle.

Behavioral analysis of tentacle motion

Movements of one or both tentacles during spinal locomotor activity (see below) were video-captured from the top in isolated preparations (n=8) with a CCD camera (Axiocam, Zeiss, Germany) with a resolution of 272×208 pixels at a rate of 20–50 frames s^{-1} either separately or in combination with the spike activity of the contralateral tentacle motor nerve. The motion trajectory of the tentacle was analyzed offline by manually tracking the distal yet still straight portion of the tentacle with respect to the base using the Vidana software (courtesy of Dr M. Hofmann). From the position coordinates, the angle of the tentacle relative to the longitudinal body axis was calculated in Matlab (MathWorks, Natick, MA, USA) and plotted as motion trajectory over time.

Electrophysiological recordings

Spontaneous or evoked locomotion in isolated tadpole preparations was recorded as bilaterally alternating rhythmic burst discharge in spinal VRs, termed fictive swimming (e.g. Combes et al., 2004). Multi-unit activity from one or two spinal VRs of segments 10–15 along with the discharge of one or both tentacle motor nerves (N=12) were recorded with individually adjusted glass suction electrodes, fabricated with a horizontal puller (P-97 Brown/Flaming, Sutter Instruments, Novato, CA, USA). The recorded neuronal activity was amplified (EXT 10-2F; npi electronics, Tamm, Germany), digitized at 10 kHz (CED 1401, Cambridge Electronic Design, Cambridge, UK), processed with commercial software (Spike 2, Cambridge Electronic Design), stored on a PC and analyzed off-line with custom-written scripts using Igor Pro (Wavemetrics, Tigard, OR, USA) or Matlab.

Data analysis

To compare the tentacle retraction on one side with the TN firing on the other side, we calculated the correlation coefficient after normalizing the angular tentacle position and the TN firing rate to their maximum for each swimming bout. The temporal relationship between rhythmic spinal VR and trigeminal motor activity was evaluated by comparing the duration of the concurrent discharge episodes, obtained from multi-unit recordings of at least one VR and one TN. These data were extracted from n=57 individual swimming episodes (these included only rhythmic alternating activity and no escape) in N=10 preparations and compared using a scatter plot and linear regression.

The degree and variation of swimming strength during fictive locomotion were estimated from integrals of the VR bursting in recordings of those locomotor episodes that showed a modulation of the swimming strength. In these traces, the swimming amplitude was calculated from integrals of VR bursts with a bin width of 30 ms (N=2 preparations) or with a bin width that matched the frame rate of simultaneously video-recorded tentacle motion (21 ms, N=3 preparations; 71 ms, N=1 preparation). Integrated and binned VR burst amplitudes were normalized to the maximal magnitude within a given swimming episode and were grouped according to amplitude into three categories (low, medium, high) indicating weak, medium and strong swimming. These three different levels were used to plot the normalized firing rate of the simultaneously recorded TN or the video-recorded tentacle position. Significance between the three levels was tested using a Kruskal–Wallis test, followed by *post hoc* multiple sample comparisons (Wilcoxon signed-rank tests).

To determine potential phase correlations between swimming (i.e. VR activity) and TN activity, the instantaneous firing rate of the VR bursts was calculated from recordings of 31 locomotor episodes in N=7 preparations. The phase relationship between the TN discharge and VR bursts was calculated and plotted as a histogram of spikes with a bin size of 5 deg (one burst cycle=360 deg). The histogram was normalized by dividing the spike count by the number of swimming cycles. Histogram data were displayed as circular plots, with their maxima aligned at 0 deg. To assess whether the distribution of spikes in the TN was phase coupled to unilateral or bilateral VR activity, a previously described procedure was employed (Kajikawa and Hackett, 2005). Briefly, the entropy (D score) of the plotted spike time histogram was calculated as the difference between unity and a normalized entropy measure (D=1−E/E_{max}). D may vary from 0 to 1, with 0 being no phase coupling (probability of firing is equally distributed over the entire swimming cycle) and 1 being maximal phase coupling, independent of the underlying spike time distribution. In order to determine the significance of D, the inter-spike intervals were reshuffled 10,000 times for each swimming episode and a D-value was calculated for each repetition (Kajikawa and Hackett, 2005). The D-value from the initial analysis was then compared with this distribution of reshuffled D-values by calculating the proportion of those that were equally large or larger than the original value. Phase coupling was considered significant if this proportion was smaller than 0.05 (i.e. one-tailed probability with α=0.05).

Anatomical identification of tentacle motoneurons

To assess the location and spatial arrangement of tentacle motoneurons in the hindbrain, fluorescent tracers (Alexa Fluor dextran 488, 546; Life Technologies, Carlsbad, CA, USA) and biocytin (Sigma-Aldrich) were applied to different portions of the trigeminal nerve in isolated *in vitro* preparations (Straka et al., 2001). Crystals of the respective tracer, melted

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to the tip of an injection needle, were inserted into various trigeminal nerve branches or the main trigeminal nerve root. After overnight incubation in oxygenated Ringer solution at 14°C, preparations were fixed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (PB, pH 7.4) at 10°C for 5–12 h and rinsed (3×10 min) in cold 0.1 mol l^{-1} phosphate-buffered saline (PBS, pH 7.4). In experiments where biocytin was applied, preparations were fixed and rinsed as described above and kept in Dent's solution [80% methanol, 20% dimethylsulfoxide (DMSO)] at room temperature for 12–14 h, transferred into 100% methanol and stored at −80°C for at least 1 h. Then, preparations were rehydrated at room temperature in 70%, 50% and 35% methanol solutions in distilled water for 1 h each, followed by 0.1 mol l^{-1} PBS (3×30 min), incubated in a 1:200 solution of streptavidin-Cy2 (Dianova, Germany) in 0.1 mol l^{-1} PBS for 2 h and rinsed in PBS (3×30 min). The brainstem of all preparations was removed, cleaned of surrounding tissue, mounted on slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA). Retrogradely labeled neurons and afferent nerve terminations were reconstructed from stacks of optical sections (1.5–3 µm) obtained from a confocal microscope (SP5, Leica, Germany). In order to map the position of retrogradely labeled motoneurons onto the rhombomeric scaffold, preparations were additionally scanned with an illumination wavelength of 633 nm, outlining the spatial arrangement of the rhombomeres (r). *Z*-axis projections, image processing and quantification of neuronal numbers were carried out using the Fiji software package (http://fiji.sc/wiki/index.php/Fiji).

Imaging of Ca^{2+} transients in tentacle motoneurons

Tentacle motoneurons were retrogradely labeled 1–2 days prior to the experiment with Calcium Green-1 dextran (Life Technologies) by applying crystals of this Ca^{2+} sensor to the peripheral part of the tentacle motor nerve. Imaging of Ca^{2+} transients was performed with a confocal scanning microscope (LSM 700, Carl Zeiss, Germany) in the presence and absence of fictive locomotor activity. To prevent potential movement artifacts during imaging, all residual muscular elements of the isolated preparation were removed, except for the most caudal portion of the tail, which was left free to perform swimming movements (see above). Images were acquired at a rate of 5–10 frames s^{-1} (ZEN black, Carl Zeiss, Germany), stored and analyzed *post hoc*. Image analysis was performed off-line using the Fiji software package and custom-written scripts in Igor Pro. Background fluorescence was subtracted and bleaching was corrected using a linear regression algorithm. All data are presented as relative changes in fluorescence ($\Delta F/F$). The duration of Ca^{2+} transients were determined as the time at half-maximal amplitude of the fluorescence change (half-width) during a given swimming episode.

Acknowledgements

The authors thank Dr M. Hofmann for the Vidana software and D. Wickmaier for contributions to initial experiments.

Competing interests

The authors declare no competing or financial interests.

Author contributions

S.H., R.B., H.S. and B.P.C. planned the experiments, S.H., R.B. and B.P.C. acquired and analyzed the data, S.H., R.B., H.S. and B.P.C. wrote the manuscript.

Funding

Financial support was provided by the German Science Foundation [CRC870 to B.P.C. and H.S.; RTG1373 to R.B.] and the Graduate School of Systemic Neurosciences [to S.H.].

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5 Discussion

5.1 Locomotion and its consequences

Locomotion not only propels an animal forward, but also has a number of consequences for the animal itself: As the animal moves forward, the visual field shifts, creating a particular optic flow. Its head movements will – if it is a vertebrate – stimulate its vestibular system in a specific, rhythmic way. Appendages such as tentacles, ears or tails might be moved along with the locomotor movements, either passively or actively. This first section of the discussion will examine locomotion and its consequences in some detail, and repeatedly compare and contrast the tadpoles and froglets examined in this thesis with other animals and their locomotor strategies.

5.1.1 Locomotor consequences are predictable

To propel themselves forward, animals might crawl, swim, walk, run, hop, trot or gallop. Amphibians differ from mammals such as humans in that they have two distinct phases of life, with different locomotor styles and usually different habitats. Tadpoles start their life in water, where they swim even as they grow legs. At the end of the metamorphosis, the tail is resorbed and all four legs are fully functional. The majority of frogs then changes habitats and spend their adult life on land, while adult *Xenopus* mostly stay in water (Nieuwkoop and Faber, 1956). Salamanders undergo a similar change from tail-based swimming to leg-based walking, but may also retain the ability of axial swimming as adults. Insects often undergo similarly drastic changes in lifestyle from crawling and swimming to flying as adults. These changes are of interest to developmental biologists, and the larvae are often studied because they are simpler and can be obtained more quickly than adults. Nevertheless it is important to bear in mind that the larvae are ‘only’ an ephemeral stage of life, and that some of their characteristics might be related to the adult lifestyle or the need to grow up quickly, rather than to their immediate surroundings. For instance, the globose body of many tadpoles, which might appear hydrodynamically inconvenient for swimming, has the advantage that it allows hindlimbs to emerge in a place where they hardly disturb the hydrodynamics of axial swimming (Wassersug, 1989).

Independent of the locomotor style, the consequences of locomotion are always fairly well predictable (Chagnaud et al., 2012), since locomotion is highly stereotyped and driven by internal central pattern generators (CPGs) which can run without sensory feedback. Indeed, this predictability of sensory consequences allows the animal to anticipate the sensory consequences, probably by using a forward model in the cerebellum, and appropriately deal with self-generated sensory inputs (see section 5.1.5). The predictability of sensory consequences also seems to be related to the speed of locomotion – the faster, the more predictable (MacNeilage and Glasauer, 2017; see section 5.1.5.1).

5.1.2 Free and fictive locomotion

Locomotion can be observed in a freely moving animal e.g. from video records, but examining the details of the neural underpinnings usually requires fictive locomotion in immobilised or curarized animals. Mostly there is at least a qualitative if not quantitative correspondence between the fictive and free locomotion. In the earliest stages of *Xenopus laevis* tadpoles that swim, locomotion has been characterised both behaviourally as well as electrophysiologically in curarized animals (Kahn and Roberts, 1982; Kahn et al., 1982; Roberts et al., 1981). The free and fictive characterisations of swimming correspond in terms of frequency, duration and other characteristics. Other examples of locomotion show a tight correspondence between the free and fictive behaviour as well, such as the propensity of tadpoles to swim (Currie et al., 2016), developmental changes from burst to beat-and-glide swimming in zebrafish (Buss and Drapeau, 2001), or escape in newt embryos (Soffe et al., 1983). Similarly, free and fictive swimming are at least qualitatively similar in terms of swimming frequency in bullfrog tadpoles (Stehouwer and Farel, 1980). The study on head movement kinematics of *X. laevis* tadpoles showed not only a qualitative but also quantitative similarity in terms of swimming frequency between behavioural and electrophysiological recordings (chapter 3, Hänzi and Straka, 2017b). Moreover, I showed a tight correspondence in developmental changes between fictive and free swimming. These and other developmental changes in locomotion will be discussed below.

5.1.3 Locomotor changes with development

First, I will recapitulate and expand on the developmental changes in locomotion of *X. laevis* described in previous sections. Since many studies describe hatchling locomotion, I can utilise these studies and combine them with the work described in this thesis to draw a more complete picture of developmental locomotor changes in *X. laevis*. Later sections will then compare and contrast different aspects of these changes with what has been described in other species.

5.1.3.1 Developmental changes of locomotion in *Xenopus laevis*

The development of *X. laevis* has been characterised and partitioned into developmental stages by Nieuwkoop and Faber (1956). The animals initially live off their yolk, and hatch at stage 37/38, which is also considered the onset of free swimming. However, while they have the ability to swim (and indeed this ability has been thoroughly scrutinized (Kahn and Roberts, 1982; Kahn et al., 1982; Roberts et al., 1981, 2010)), initially they spend 99% of their time immobile and attached to a substrate (Jamieson and Roberts, 2000). When they become dislodged, they swim again until they encounter an object and re-attach themselves using their cement gland (which they posses at stages 26 - 46, Nieuwkoop and Faber (1956)). The stopping of these tadpoles in response to touch has been characterised both at a behavioural and at a neural level (Boothby and Roberts, 1992; Li et al., 2003; Perrins et al., 2002). The propensity to swim markedly increases at the start of feeding at stage 46 (Currie et al., 2016). Then the animals continue a similar lifestyle as they grow trunk and tail in the premetamorphic stages (46 - 54), and as the limbs grow quickly during prometamorphic stages (54 - 58; classification according to McDiarmid and Altig (1999)). The metamorphic stages (59 - 66) are characterised by the emergence of forelimbs and finally the resorption of the tail – a stage 66 animal is essentially a small adult in form.

Not only does the behaviour change with development, but so do the locomotion and the underlying central pattern generator (CPG). The changes in swimming frequency will be highlighted here, beyond the developmental stages that were examined in the head kinematics study (see chapter 3, Hänzi and Straka, 2017b); these changes along with the most important landmarks of development are shown in Figure 7.

Very brief and mostly irregular episodes of 'swimming' can already be elicited before hatching/the free swimming stage. These short episodes have a frequency of 3 - 5 Hz at stage 27, and get longer and increase in frequency (10 - 20 Hz) by stage 29/30 (black bars in Fig. 7; van Mier et al., 1989). At the time of hatching, or at the final embryonic stages (stage 37/38), both fictive and free swimming can be elicited; its frequency ranges from 10 to 25 Hz, and decreases within a given episode (Kahn et al., 1982; Sillar and Roberts, 1993). At that time, there is a single impulse in each ventral root for each swim cycle; by stage 41, when the embryo has become a larva and has finished its first day of life, there is a burst of impulses in each cycle (Sillar et al., 1991). Additional parameters have also been examined at this stage (Roberts et al., 1981; Soffe and Roberts, 1982a, 1982b; Soffe et al., 2009; Tunstall and Sillar, 1993), such that it is one of the better-described vertebrate CPGs to date. After the onset of feeding, which is accompanied by an increased swimming propensity, the basic pattern of swimming is established, but the animal needs to grow and adjust its swimming and CPG accordingly. I described the swimming frequencies from stage 46 to 54 in the kinematics study (chapter 3, Hänzi and Straka, 2017b). They are spread around 8 Hz for stage 46 - 48

tadpoles, around 6 Hz for stage 50 - 52 tadpoles, and around 5 Hz for stage 53 - 54 tadpoles (see black bars in Fig. 7). Other authors have grouped stage 50 - 54 tadpoles and reported swimming frequencies of 3 - 5 Hz (Combes et al., 2004). At stage 58, the swim and kicking rhythms interact, but by stage 60/61 they are at least somewhat independent, at around 2 Hz for swimming and 0.6 Hz for kicking (Combes et al., 2004). At stage 65, the tail is resorbed, so only the kicking remains – around frequencies of 0.9 Hz (blue bars in Fig. 7, Combes et al., 2004).

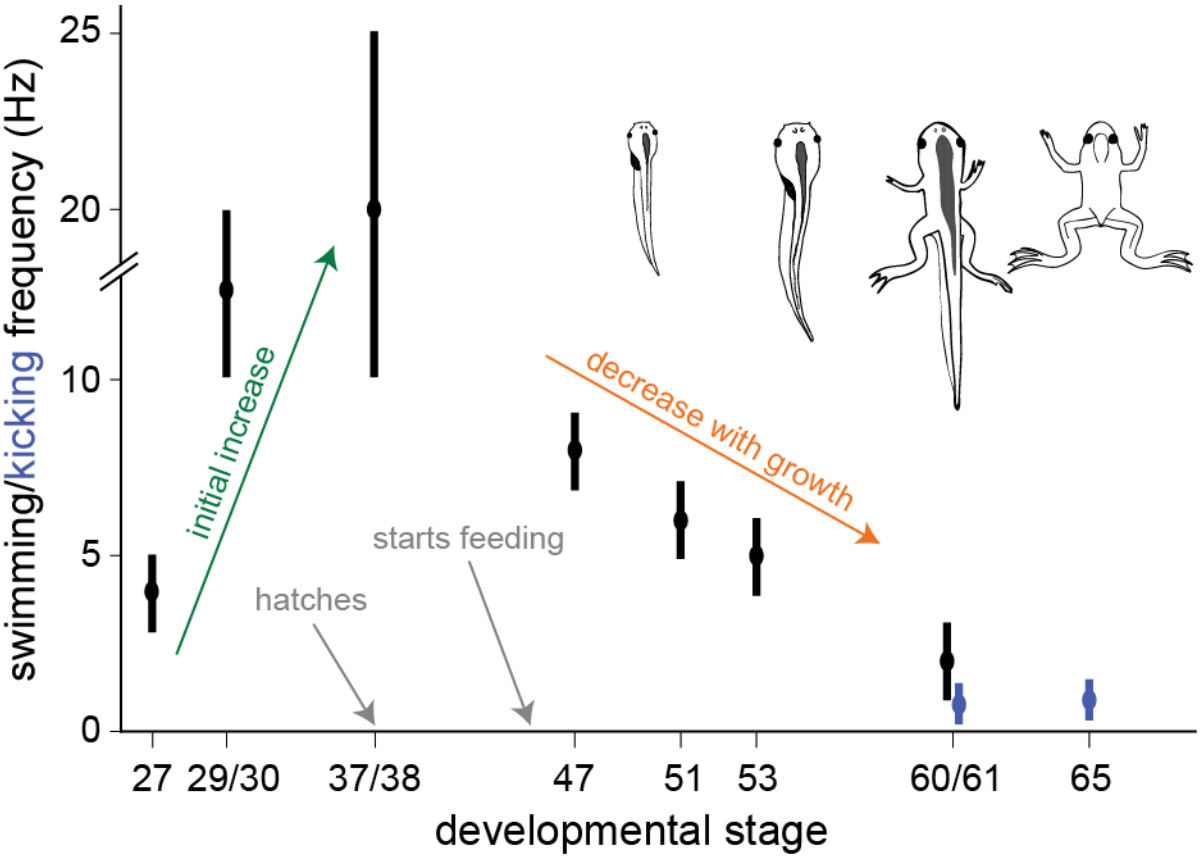

Figure 7. Changes of swimming frequency with development in *Xenopus laevis*. This graph illustrates the swimming frequency against developmental stage. The frequency values are indicated as black/blue bars and are approximate values based on the literature (Combes et al., 2004; Hänzi and Straka, 2017b; Kahn et al., 1982), the animal schemes are taken from Hänzi and Straka (2016a). See text for further explanation.

5.1.3.2 Comparison to other animals

Since the developmental stages employed above to describe the progress of development are particular to *X. laevis*, other measures of development are needed for comparisons to other species. In fish, the total length is the best quantitative measure of developmental progress (Fuiman and Webb, 1988); it is a better indicator of development than age (Higgs et al., 2002). The speed of growth depends on temperature as well as animal density (Brunkow and

Collins, 1996), making age susceptible to these confounding factors. This is why the development of *X. laevis* was characterised in terms of total length in the study on head movement kinematics (chapter 3, Hänzi and Straka, 2017b).

5.1.3.2.1 Hydrodynamic considerations

Since tadpoles swim in water, they have to deal with hydrodynamics. The most drastic change in hydrodynamics is the change from a viscous regime at low Reynolds numbers (Re), in which laminar flow dominates, to an inertial regime at high Re, in which turbulent flow dominates. There is also an intermittent regime, but the exact boundaries are defined differently by different authors (Fuiman and Webb, 1988; McHenry and Lauder, 2005). As the larvae hatch, they probably face a viscous regime, but they 'grow out' of that rather quickly. Larval anchovy, for example, change from burst to beat-and-glide swimming, which is hydrodynamically more efficient as they grow out of the viscous regime (Weihs, 1980). For zebrafish, the most dramatic changes in hydrodynamics occur as the animals grow from 5 to 15 mm in length (Fuiman and Webb, 1988). This likely is similar for the *X. laevis* tadpoles, so the vast majority of animals employed in the study on kinematics (chapter 3; stage 46 - 56, 10 - 45 mm total length) have grown out of the initially viscous regime already. It is possible that almost from the very start of the feeding stage at stage 46, when the animals start to swim more often, they do not face a viscous environment anymore. Behaviourally, both *X. laevis* and other larvae, such as angelfish or anchovy, are similar in that they are able to swim after hatching but spend most of their time resting (Hunter, 1972; Jamieson and Roberts, 2000; Yoshida et al., 1996). Zebrafish similarly increase the time they spend moving from 3 to 7 days post fertilisation (dpf; Farrell et al., 2011). This initial resting might let the animals grow out of the viscous regime by the time they start swimming more often, such that they do not need to face a fundamental change in hydrodynamics.

5.1.3.2.2 Changes in locomotor frequency with development

As illustrated in Figure 7, there are two main changes in swimming/locomotor frequency during the development of *X. laevis*: An initial and rapid increase that occurs before the free swimming stage/hatching (van Mier et al., 1989), and a subsequent decrease in swimming frequency with growth (chapter 3, Hänzi and Straka, 2017b). Since there is a plethora of studies on the development of the swimming capability, many of the examples of frequency changes with development are probably similar to the first increase in frequency in *X. laevis* tadpoles. Only very few studies have looked at subsequent changes with growth once swimming has been established.

At swimming onset, the tadpoles exhibit one spike per swimming cycle in their ventral roots, but this changes over the first day to become a burst (Sillar et al., 1991). The same is true for zebrafish from 3 to 5 dpf (Thirumalai and Cline, 2008). Similarly, the very early increase in swimming frequency in *X. laevis* (van Mier et al., 1989) is mirrored in zebrafish, where the frequency increases from about 7 Hz at 27 hours post fertilisation (hpf) to about 28 Hz at 36 hpf (Saint-Amant and Drapeau, 1998). In the moth *Manduca sexta*, rhythmic locomotor-like patterns can be observed in the three days before eclosion (i.e. when the adult animal emerges from the pupa), and over these three days, the frequency of these rhythms increases (Kammer and Kinnamon, 1979). For a number of animals, an increase in locomotor frequency has been recorded during the first two to three weeks after birth or the start of adult life. For instance, rats increase the swimming frequency over the first two weeks after birth (Bekoff and Trainer, 1979), and the flapping rate of chicken increases during the first two weeks after hatching (Provine, 1981a). Similar increases in flight frequency occur in insects, such as in the desert locust, which increase their flight frequency exponentially from 10 to 20 Hz during the first 3 weeks of adult life (Kutsch, 1971), or in the Australian plague locust, where the flight frequency also increases in the first three weeks (Altman, 1975). It is unclear whether this slower increase over weeks is similar to the early and fast increase of swimming frequency before hatching in tadpoles, whether it is similar to the second, much slower decrease with growth even though the change is in the opposite direction, or whether it is a different process altogether. I would guess that – at least for the case of the chicken, whose wings are too small to allow sustained flight – the change is similar to the initial increase in *X. laevis* tadpoles, since it also occurs before the behaviour is fully functional. The increase in frequency in locusts and moths might be different as flight is already functional, and the role of sensory feedback also differs (see below).

5.1.3.2.3 The role of sensory feedback

In some of the frequency changes with development described above, experimenters have removed sensory feedback during the time when the changes normally occur, and have then checked whether the change still occurred without feedback. In fact, this was the case in all instances: The increase in wing flapping in chicken occurs even if the animals are prevented from flapping, in featherless mutants, and if the wings are amputated (Provine, 1979, 1981a, 1981b). In the desert locust, the increased flight frequency does not depend on flight experience either (Kutsch, 1971). *Xenopus* tadpoles have also been raised in an immobilizing solution, but nevertheless developed fairly normal swimming up to stage 45 (Haverkamp, 1986; Haverkamp and Oppenheim, 1986). The initial frequency increase in *X. laevis* and the increase in chicken and desert locusts therefore occur without any practice and in the absence of sensory feedback. To what extent the same is true for the later and very much slower

decrease of swimming frequency in *X. laevis* tadpoles remains to be determined. Acute removal of sensory feedback for the recording of fictive swimming does not markedly change the swimming frequency, which still is appropriate for the animal's developmental stage (chapter 3, Hänzi and Straka, 2017b). This differs from locusts, where acute destruction of the sensors providing the feedback leads to an immediate drop in flight frequency (Kutsch, 1971, 1974). The neural mechanisms behind the frequency changes in locusts therefore most likely differ from the mechanisms occurring in *X. laevis* tadpoles, and whether these in turn are similar to chicken remains unclear. None of these studies have explicitly examined changes in frequency with growth of the animal well after the locomotor behaviour became established. Only one study incidentally showed that larger fish tend to swim at lower frequencies than smaller fish of the same species (Bainbridge, 1957). Comparing across species, one can also imagine that a pony trots at a higher frequency than a horse, or a mouse runs at a higher frequency than a rat. It remains to be elucidated whether a decrease in frequency with increases in size is a general principle observable within and across species, though it has been noted previously that when moving at the same speed, larger animals generally take longer strides at lower frequencies (Alexander, 1984). Furthermore, it is currently unknown to what extent changes in biomechanics accompanying growth drive changes in locomotor frequency. The CPG might adapt do biomechanical changes, and/or might change intrinsically. Such changes could include but are not limited to changes in cellular properties of the neurons in the CPG, or changes in synaptic connections and strength (Selverston, 1980). Teasing apart biomechanical from intrinsic factors while elucidating the mechanisms of adaptation at the circuit level will require careful experiments over a long developmental timeframe.

5.1.4 Motor consequences of locomotion

As animals swim, run or trot, they not only move those parts of their bodies that generate the forward thrust, but also move body parts not involved in the locomotion, such as appendages. For instance, salamanders, axolotl or alligators retract their legs when they swim (D'Août and Aerts, 1997; Delvolvé et al., 1997; Fish, 1984). In the third study presented in this thesis (chapter 4, Hänzi et al., 2015), I examined the movements of tadpoles' tentacles during swimming, and found that they were consistently retracted. Before discussing the mechanism behind this as well as potential functions of such coupled movements, I will introduce the tadpoles' tentacles in more detail.

5.1.4.1 Tentacles of *Xenopus laevis*

Tadpoles of *X. laevis* of certain developmental stages possess a pair of mobile appendages that protrude from the corners of their mouth. Tentacle buds are already present before stage 50, but become only appreciably larger (extending further than their mouths) around stage 53.

These tentacles consist of a cartilaginous rod at the centre, surrounded by skin (Ovalle et al., 1998). Around metamorphic climax, the cartilage is resorbed, the tentacles become floppy and finally disappear entirely by the time the animals turn into frogs (Nieuwkoop and Faber, 1956). The full length of the tentacles has previously been described as 2.5 - 4 cm (Ovalle et al., 1998), but the animals from our breeding developed tentacles with a maximal length of 1 cm, and many animals failed to develop more than tentacle buds (see e.g. chapter 2).

The function of these tentacles has not yet been resolved. Early reports hypothesised that tentacles might be necessary for balance (Brown, 1970). However, animals do not have compromised balance before they develop or after they lose tentacles, and animals that never develop tentacles are able to swim normally (chapter 2, Hänzi and Straka, 2017a). Some studies described the presence of Merkel cells in the tadpoles' tentacles (Eglmeier, 1987; Ovalle, 1979), suggesting a tactile function. These Merkel cells are present at a density that might well be similar to that of the surrounding skin (Nurse et al., 1983), and no other specialised sensory structures have been found in detailed electron microscopy studies (Eglmeier, 1987; Ovalle, 1979; Ovalle et al., 1998). This contrasts with tentacles or barbels in fish, which often contain gustatory receptors (Bhatti, 1952; LeClair and Topczewski, 2010; see Fox, 1999 for a review), and some are even an outgrowth of the gustatory system (McCormick, 1993). Some other tadpoles also possess tentacles, but these do not contain a stiff cartilaginous centre (Orton, 1943), and therefore are unlikely to serve the same function. Some other amphibians – caecilians – possess tentacles too (Burger et al., 2007). However, in their case in addition to the environment and the ecology being widely different, their tentacles are associated with their vomeronasal organ (Billo and Wake, 1987), and therefore are unlikely to be homologous or even analogous in function.

The tentacles of *X. laevis* tadpoles are therefore unlike gustatory fish barbels, and likely serve a tactile function (see Fig. 8 for example mechanosensory responses). Physiologically, responses to tactile stimulation have been mentioned only in preliminary reports (Cannone and Kelly, 1977). Unpublished observations from my work also suggest that these tentacles respond to touch. I recorded neural activity from the two sensory nerves entering the tentacle (see Fig. 8B), while touching the tentacle lightly with a glass rod mounted on a piezo stimulator (Fig. 8C). These afferents responded briefly to touch, but when the touch stimulus was sustained, the response was not. This is in line with amphibian Merkel cell-associated somatosensation being fast-adaptive (Mearow and Diamond, 1988). A very strong water jet from a pipette directed at the tentacle could also evoke brief responses, but slower water movements did not evoke responses. The somata of the sensory neurons are located in the trigeminal ganglion, and projections can be found at almost all levels of the hindbrain (data not shown).

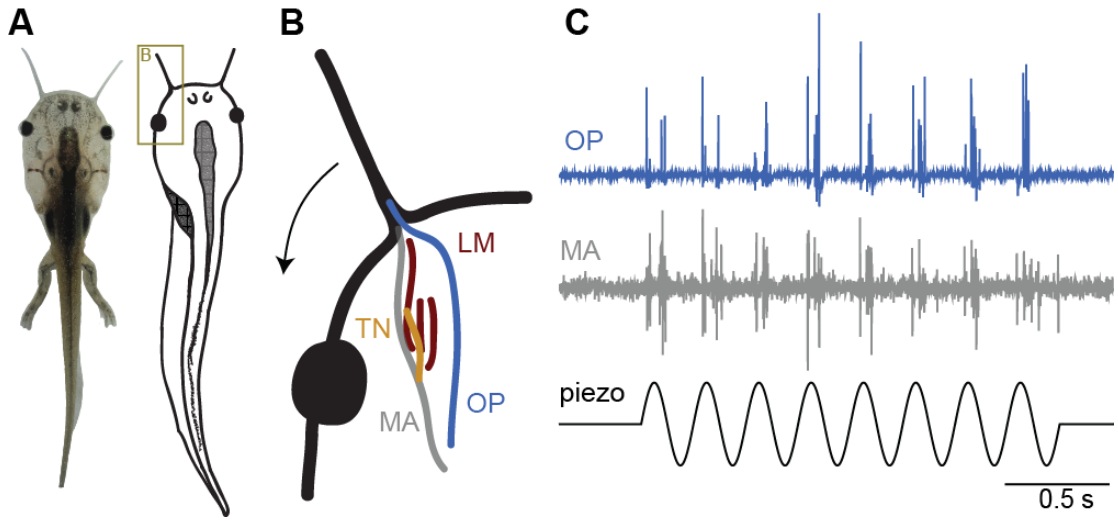

Figure 8. Tentacles of *Xenopus laevis* tadpoles are mechanoreceptive. A) Image and scheme illustrating tadpoles with tentacles (from Hänzi and Straka, 2016a, 2016b). B) Schematic inset depicting the tentacle and its innervation. The red part represents the levator mandibulae (LM) muscle; its lateral part inserts at the base of the tentacle, and contraction of the muscle retracts the tentacle (direction indicated by black arrow). The LM is innervated by a branch of the mandibular nerve termed tentacle nerve (TN, orange). The sensory innervation of the tentacle comes from both a branch of the ophthalmic (OP, blue) and a branch of the mandibular (MA, grey) nerves, which are in turn subdivisions of the trigeminal nerve. C) Example recording illustrating mechanosensitive responses. The OP and MA were recorded extracellularly at the base of the tentacle, with the afferent part in a glass suction electrode. The tentacle was stimulated by touch from a piezo stimulator, whose approximate movements towards (upwards deflection, touches the tentacle) and away from the tentacle (downward deflection) are shown in the bottom trace.

Overall, the data from my unpublished observations together with the findings on Merkel cells (Eglmeier, 1987; Nurse et al., 1983; Ovalle, 1979; Ovalle et al., 1998) strongly suggest that the tentacles are used for mechanosensation. However, tactile responses are brief and not sustained, and likely do not provide much detail. They might simply inform the animal that something is in front of it, and the information content likely is much more similar to a blind person's stick than to a rat or mouse whisker. Probably tadpoles' tentacles are still simpler than catfish mechanosensory barbels, which are used for prey detection (Biedenbach, 1971), but potentially similar to the mechanosensory tentacles of aquatic snakes that prey on fish (Catania et al., 2010). While the tadpoles might well use these tentacles for wall following or other touch-related information gathering, animals without tentacles equally follow the walls (chapter 2, Hänzi and Straka, 2017a). Tentacles are therefore not necessary for these behaviours and likely do not represent very specialised structures. When absent, the animals might use their facial skin to gain tactile information about their environment.

5.1.4.2 Appendage movements during locomotion

The tentacles of *X. laevis* tadpoles are extended forward at rest and when the animal is slowly cruising with only the tip of the tail undulating (Hoff and Wassersug, 1986). However, when

the animal swims, it retracts its tentacles laterally. These movements were examined in detail in the third study of this thesis (chapter 4, Hänzi et al., 2015). A number of points are worth repeating: The left and right tentacles move together, and the motor command for retraction on one side is positively correlated with tentacle movement on the other side. The motor neurons responsible for the retraction have their somata in rhombomeres 2 and 3 of the hindbrain, and send out their axons in the mandibular branch of the trigeminal nerve to the lateral part of the levator mandibulae muscle, which retracts the tentacle. There is no antagonist – forward motion of the tentacle is achieved passively, probably by some sort of cartilaginous spring at the base of the tentacle. The duration of both the motor commands responsible for tentacle movements as well as the tentacle movements themselves are highly correlated with the duration of swimming activity – the tentacles are retracted during swimming, and not before and not after. Moreover, both the motor commands and the tentacle movements vary in amplitude with the strength of (fictive) swimming. And while other corollary discharges from the tadpole's swimming have been shown to be strictly phase-coupled (Combes et al., 2008; Lambert et al., 2012), some motor units show some phase coupling to the swimming rhythm while others do not. These motor neurons might integrate a phasic corollary discharge (CD) from the spinal cord, and/or the CD might be supplemented by tonic midbrain signals.

Having described the tentacle movements and their coupling to swimming in some detail, we can move from mechanisms to function and ask about the survival value: Why do the animals retract their tentacles while swimming? In previous paragraphs I described the probable function of tentacles as tactile probes. With the tentacles retracted during swimming, the animals are not able to acquire tactile information from the tentacles about the space in front of them. While this might seem wasteful, there are a number of potential reasons why retracting the tentacles during swimming could in fact be advantageous, and none of them are mutually exclusive. First, in many instances, these tadpoles swim to escape. Unlike zebrafish larvae, which catch moving prey and therefore need at least visual feedback during swimming (Gahtan et al., 2005), *X. laevis* tadpoles feed while cruising with only the tip of their tail moving (Hoff and Wassersug, 1986). When they engage their whole tail to swim, the function probably is to escape, and they do not need to gain information about where they are heading – they simply want to get away from their current location. Second, if the tentacles were extended during swimming, they would be exposed to water flowing over them at high velocities. While the preliminary sensory experiments described above showed that a very directed and fast water current is needed to elicit responses, this might well be the case given the very high head angular velocities of up to 1000°/s in the larger animals with tentacles (chapter 3, Hänzi and Straka, 2017b). Third, the tentacles might be damaged if extended. The tentacles can reach up to about 20% of the body length (chapter 4, Hänzi et al., 2015), but are only 0.3 - 0.4 mm in diameter (Ovalle, 1979), and might therefore be damaged

during the high-velocity left-right oscillations of the head. Fourth, retracting the tentacles might be hydrodynamically advantageous and reduce drag. In an amphibious robot swimming in a salamander-like fashion, retracting the legs during swimming increased swimming speed by 39% compared to extended legs (Crespi et al., 2013). Similar hydrodynamic effects might be at play in limbed animals that retract their legs during swimming, such as alligators (Fish, 1984; Manter, 1940), salamanders (Delvolvé et al., 1997) and lizards 'swimming' in sand (Maladen et al., 2009, 2011). A similar effect might be the reason that larval zebrafish actively retract their pectoral fins during fast axial swimming (Green and Hale, 2012). At least for large appendages, a hydrodynamic advantage of retraction is therefore very likely. These different potential reasons for retracting small appendages might similarly apply to gill retraction during swimming in axolotl (D'Août and Aerts, 1997) or tentacle retraction in aquatic snakes as they strike prey (Catania, 2009; Catania et al., 2010).

5.1.4.3 Coupling different motor behaviours

A potential issue when describing coupled motor behaviours is the terminology – we described tentacle retraction as being caused by CD from the spinal cord. Whether this should really be called CD is to some extent debatable, because CD commonly influences sensory processing. However, some motor effects of CD have also been previously described, such as eye movements during swimming in tadpoles (Lambert et al., 2012), or middle ear muscle contractions with vocalisations (Borg and Allen Counter, 1989; Suga and Jen, 1975), although these have obvious advantages for sensory processing. Moreover, some authors have argued for an explicitly broad definition of CD: "We suggest use of 'corollary discharge' as a broad term to encompass neural signals that are generated in motor centres and that are not directly used to generate the ongoing motor activity. Often they act to modulate sensory processing. A broad term is useful because corollary discharges have diverse properties, targets and functions." (Poulet and Hedwig, 2007). Using the term CD for the command that retracts appendages during swimming therefore seems justified. Indeed I would predict that a similar central mechanism also applies for instance to gill retraction during swimming in axolotl (D'Août and Aerts, 1997).

In other cases of motor-motor coupling, the mechanism might well be more complicated. In the study on tentacle retraction during swimming (chapter 4), we found some evidence for a tonic signal during swimming, which might either mean that the motor neurons integrate the phasic spinal CD, or that there is a tonic signal e.g. from midbrain locomotor centres, or both. It becomes even more complicated when two CPGs are potentially involved. Adducting legs during swimming in alligators, axolotl or salamanders (D'Août and Aerts, 1997; Delvolvé et al., 1997; Fish, 1984) might still be directed by a CD from the spinal centres that produce the undulatory swimming. However, the legs also have their own CPGs that direct walking.

Whether the swimming CPG can bypass or overrule the walking CPG remains to be determined. Similarly, two CPGs must be coupled when respiration and locomotion occur with a phase-coupled relationship (Bramble and Carrier, 1983). Such coupling observes a fairly strict phase relationship in hopping wallabies (Baudinette et al., 1987) or running horses (Young et al., 1992), but is more variable in human runners (Bramble and Carrier, 1983). These reports have only considered biomechanical reasons for coupling; however, I would predict that some central neural coupling is also present. This has indeed been reported in preliminary form for respiration and swimming in tadpoles (Combes et al., 2015). Whether such a neural linkage is a 'pure' CD from the locomotor centres, a top-down signal e.g. from midbrain locomotor centres to both CPGs, or a combination of both is still an open question. In some bats, wing-beats, respiration and ultrasonic calls are all coupled (Suthers et al., 1972; Wong and Waters, 2001) – in that case, there are three different types of CPG-directed movements to be coordinated.

More generally, different (motor) behaviours need to be coupled appropriately, and incompatible movements must be prevented. In the gastropod mollusc *Pleurobranchaea*, for instance, escape and swimming inhibit feeding via those neurons that trigger swimming (Jing and Gillette, 1995). In the same animal, there is reciprocal inhibition between feeding and withdrawal from tactile stimulation, which is mediated by a pair of identifiable CD neurons from feeding that inhibit the withdrawal reflex (Kovac and Davis, 1980a, 1980b). These different behaviours and their relationships have led to the concept of behavioural hierarchies (Davis, 1979; Davis et al., 1974). However, in many of the examples described above, the relationship is unlikely to be hierarchical, and the interaction requires coordination rather than suppression. Only very few of these interactions have been described at the behavioural level, and even fewer of the neural mechanisms underlying the coordination are known – leaving plenty of scope for future research. In tadpoles of *X. laevis*, for example, one might examine how filter-feeding, breathing and locomotion interact, and how this interaction is influenced by external stimulation.

5.1.5 Sensory consequences of locomotion

Tentacle retraction during swimming is a somewhat unusual example of CD – one with motor consequences. Classically, CD influences sensory processing. CDs can target different levels of sensory processing (Fig. 1 in the introduction) and can have different effects depending on the meaning of the reafference. These varieties have been classified into lower- and higher-order by Crapse and Sommer (2008). Lower-order CDs serve to inhibit reflexes and filtrate sensory inputs, whereas higher-order CDs serve sensory analysis and stability as well as sensorimotor planning and learning. Reflex inhibition by CD has been demonstrated in tadpoles of *X. laevis* (Li et al., 2002; Sillar and Roberts, 1988) as well as in crayfish (Kuwada and Wine, 1979). Sensory filtration can help to suppress unwanted reafference, for instance in

the lateral line of dogfish and *Xenopus* tadpoles (Chagnaud et al., 2015; Russell and Roberts, 1974) or in the cricket auditory system (Poulet and Hedwig, 2006). More sophisticated analysis of sensory inputs and specific as well as plastic cancellation of reafference such as in weakly electric fish (Bell, 1989) are classified as higher-order CD. Similarly, maintaining sensory stability across movements such as gaze shifts and saccades with the help of CD (Sommer and Wurtz, 2002) is considered a higher-order CD. The definitions between lower- and higher-order CD become somewhat blurry when sophisticated analysis is applied early in sensory processing, such as in the suppression of visual reafference during saccades in flies (Kim et al., 2015, 2017). Similarly sophisticated mechanisms are at play to deal with vestibular reafference in monkeys (Brooks and Cullen, 2014; Carriot et al., 2013), which will be discussed in more detail below.

5.1.5.1 Vestibular consequences of locomotion

During locomotion, head movements accompany the locomotor movements not only in tadpoles, where the head and the trunk are fused, or in anguilliform fish or snakes, which undulate, but also in mammals moving on land. Horses, for instance, show characteristic head movements phase-locked to the locomotor cycle (Chagnaud et al., 2012). These head movements represent self-generated sensory inputs to the vestibular system. To be able to judge the strength of these self-generated inputs, knowing parameters such as amplitude and frequency of the head movements, or even better the statistics of frequency and amplitude, is important. While our study on head movement kinematics focused on those head movements generated during undulatory swimming (chapter 3, Hänzi and Straka, 2017b), other studies have more broadly examined the statistics of natural head movements in humans, monkeys and mice (Carriot et al., 2014, 2017a, 2017b). In larger animals such as mice and monkeys, head movements can be recorded by attaching a miniature accelerometer to the head, whereas studies in smaller and/or aquatic animals have generally relied on video tracking to extract head movements. This is what I did for tadpoles of developmental stage 46 – 56 (chapter 3, Hänzi and Straka, 2017b). Previous reports had established that lateral movements are present along the whole body of the anuran tadpoles during swimming (Kahn et al., 1982; Wassersug and Hoff, 1985). While the lateral excursions are smallest at the level of the otic capsule (Wassersug and Hoff, 1985), in stage 37/38 *X. laevis* tadpoles the smallest excursion near the centre of the head is still considerable (Kahn et al., 1982). These left-right head oscillations are therefore very likely to stimulate the animal's own vestibular system in addition to being necessary for generating thrust (Liu et al., 1997).

In my study on head kinematics of *X. laevis* tadpoles (chapter 3, Hänzi and Straka, 2017b), the animals' head movements during swimming reached median angular velocities of 150 - 600°/s, with the maxima going up to 2500°/s in small tadpoles (below stage 50, 10 - 15 mm total length), and 1000°/s in large tadpoles (stage 52 - 54, 35 - 40 mm total length). These

values are higher than the maximal angular velocities reached by spinning dolphins or bucking cattle (200 - 600°/s depending on the plane; Kandel and Hullar, 2010). Mice reach up to 1300°/s angular velocities, and macaque monkeys up to 1500°/s (Carriot et al., 2017a). Zebrafish larvae, on the other hand, move their heads at higher angular velocities than *X. laevis* tadpoles; they can reach 10000°/s during routine swimming and up to 32000°/s during escape (Budick and O'Malley, 2000). The differences between *Xenopus* tadpoles and zebrafish larvae, as well as the changes with development in tadpoles, are likely related to the swimming frequency: This parameter declines with development in tadpoles, while head movement amplitudes do not change systematically. Similarly, zebrafish larvae swim at much higher frequencies (25 - 40 Hz during slow swimming, and 45 - 75 Hz during burst-like swimming (Budick and O'Malley, 2000)), compared to the 4 - 10 Hz observed in *X. laevis* tadpoles. This difference in frequency probably is the main reason for the differences in angular velocity. The decline in swimming frequency with development in *X. laevis* tadpoles is likely to be the main driver for the decline in angular velocities with development. In addition to these changes, small and large tadpoles also differ in the location of the axis of rotation of the head movements (Lambert et al., 2009). According to these authors, larger tadpoles (stage 57) move their heads in such a way that the axis of yaw rotation is in the centre of the head. This means that the animal stimulates its own sensory system with high angular acceleration components and very little left-right linear motion components (in addition to the linear forward movement). Small animals (stage 47), on the other hand, oscillate their head from left to right in a way that generates additional linear forces, because the axis of yaw rotation is outside the head. In addition to changes in strength of angular vestibular self-stimulation during swimming with tadpole growth, which I demonstrated in the kinematics study (chapter 3, Hänzi and Straka, 2017b), changes in the presence and strength of linear vestibular self-stimulation (Lambert et al., 2009) therefore also occur during tadpole growth.

5.1.5.1.1 Relationship to the development of the vestibular system

The vestibular organs in *X. laevis* develop from simple sacs at stage 28 to miniature versions of the adult organ at stage 52 (Bever et al., 2003; Nieuwkoop and Faber, 1956). In tadpoles as well as in fish, static vestibulo-ocular reflex (VOR) responses based on sensing gravity appear immediately after hatching and before angular VOR responses (Beck et al., 2004; Horn et al., 1986). The semicircular canals (SCCs) are formed from two outpocketings that then fuse. In *X. laevis*, this fusion occurs at about stage 46 for the horizontal canal and between stage 46 and 47 for the anterior and posterior canals (Haddon and Lewis, 1991). The tadpoles can therefore not sense rotations with their vestibular system before they reach these stages. However, the neural circuitry underlying the VOR is already functional when the

SCCs fuse (Lambert et al., 2008), but the canals are still very small. The whole otic capsule doubles in antero-posterior length from stage 45 to stage 47, and again from stage 47 to 50, reaching about 1 mm at stage 50 (Bever et al., 2003; Quick and Serrano, 2005). It is therefore reasonable to ask, given that smaller canals are less sensitive and lumen diameter probably imposes a lower limit for a functional SCC (Muller, 1999), whether these small canals are already functional. This was done by Lambert and colleagues, who looked for angular VOR responses in animals of stage 42 - 52. Angular velocities up to ±60°/s with angular accelerations up to about 400°/s^2 were employed, and with such a stimulus, VOR responses could not be elicited before stage 48. However, my kinematic study showed that animals around stage 46 - 48 move their heads during swimming at much higher accelerations than 400°/s: Median values ranged up to 20000°/s^2, and maximal values up to 250000°/s^2 (chapter 3, Hänzi and Straka, 2017b). It is likely that such stimuli, which – using only the median – are 50 times larger than the ones tested with passive external stimulation, are strong enough to elicit afferent responses even though the canals are still very small. Therefore both small animals (below stage 50), which have smaller SCCs but move their head at higher angular velocities, as well as larger tadpoles (stage 50 until metamorphosis), which have larger and therefore more sensitive canals but move their head at lower angular velocities, very likely stimulate their SCCs when swimming (in particular the horizontal SCC; see above for additional linear components). Therefore, all stages have to deal with vestibular reafference during swimming. Some cancellation occurs in the periphery in animals of developmental stage 48 - 55 via the efferent system (Chagnaud et al., 2015). Likely, the mechanisms that deal with reafference are already in place as soon as the animal starts swimming and has complete horizontal canals at stage 46 (Currie et al., 2016; Haddon and Lewis, 1991). Moreover, the locomotor CD described in the later stages, which directs appropriate compensatory eye movements during swimming (Combes et al., 2008; Lambert et al., 2012; von Uckermann et al., 2013), is probably already present in smaller animals.

5.1.5.1.2 Dealing with vestibular consequences

Vestibular stimulation can elicit a number of reflexes, such as the VOR, which stabilizes gaze in response to unexpected perturbations, or the vestibulo-spinal reflex, which stabilizes posture. Whether these reflexes are helpful or detrimental during locomotion and voluntary movements depends on the context: They might well oppose the intended movement. In the case of gaze stabilization during swimming in tadpoles, the VOR could conceivably be used to stabilize gaze. However, at least in *X. laevis*, it is not sensory feedback that drives compensatory eye movements during swimming, but locomotor CD from the spinal cord (Combes et al., 2008; Lambert et al., 2012; von Uckermann et al., 2013). A locomotor CD crosses in the spinal cord and then ascends to the hindbrain, where it directs eye movements

that compensate the left-right head oscillations during swimming (Lambert et al., 2012). With metamorphosis and the accompanying change in locomotor style, the effect of the CD also changes to elicit appropriate vergence movements (von Uckermann et al., 2013). The authors have explicitly shown that it is not vestibular sensory feedback driving these compensatory eye movements, and that additional passive yaw vestibular stimulation is suppressed, while roll can still elicit a VOR (Lambert et al., 2012). Therefore, specific mechanisms must be present to deal selectively with vestibular reafference (i.e. the vestibular feedback generated by strong head movements during swimming). The self-generated horizontal canal input is suppressed to prevent a horizontal VOR. The roll VOR, which can still be elicited, might be caused by stimulation of the vertical canals or the lagena and the utricle, which are also stimulated, since the gravity vector changes direction during roll. It remains to be elucidated how specific the locomotor CD is in dealing with vestibular reafference: One possibility is that all canal-related inputs are suppressed and otolith-related inputs are not. Another possibility is that only horizontal canal-related inputs are suppressed. While these questions have yet to be answered, it has been shown that some suppression already occurs in the periphery by action of the efferent system (Chagnaud et al., 2015). The efferent system projects to both hair cells and afferents; in amphibians and fish, efferents are shared between the lateral line and different vestibular endorgans (Birinyi et al., 2001; Hellmann and Fritzsch, 1996). The overall effect of efferent activity is to suppress afferent encoding in both modalities. This is similar to the suppressive effect of efferent activity on the lateral line system in adult *X. laevis* (Russell, 1968), in dogfish (Russell and Roberts, 1972), or toadfish (Tricas and Highstein, 1990, 1991). In *Xenopus* tadpoles, the effect of efferents on vestibular afferents is similarly suppressive: It reduces the gain of the VOR by about 30%, from 0.6 to 0.4, although individual afferents might become more or less active with efferent activity (Chagnaud et al., 2015). This contrasts with the lack of effect of the efferent system on vestibular afferents in macaque and rhesus monkeys, where both otolith afferents and semicircular canal afferents respond equally to passively applied vestibular stimulation and active vestibular stimulation resulting from voluntary head movements (Cullen and Minor, 2002; Jamali et al., 2009; Sadeghi et al., 2007). This difference in dealing with vestibular reafference at the level of the periphery might be caused by a number of factors: By the different types of movements (highly trained voluntary movements in monkeys vs. rhythmic locomotion in tadpoles), by differences in the function of the efferent system related to the presence/absence of a lateral line system, by differences in behavioural strategies related to the presence/absence of a neck and voluntary neck movements, or by a combination of these aspects. In particular, the efferent system might well have different functions in primates compared to other animals, such as guidance in development or maintaining the long-term symmetry between the two labyrinths (see discussion in Cullen and Minor, 2002).

These differences in processing might well continue at more central processing stages. However, comparisons are not yet possible since the effects of CD on these central processing stages have not been described in tadpoles. Future investigations will hopefully shed light on this. Nevertheless, the further processing of vestibular reafference in monkeys will be described below, since it is the only system where it has been examined in great detail.

As already mentioned, vestibular afferents in monkeys respond equally to passive (external) and active (self-generated) vestibular stimulation (Cullen and Minor, 2002; Jamali et al., 2009; Sadeghi et al., 2007). In second order vestibular neurons in the vestibular nucleus (VN), there are different populations of cells that are differentially modulated. The neurons representing the central leg of the VOR (position-vestibular-pause neurons) respond equally to active and passive vestibular stimulation, but their activity is modulated by gaze efference copy (Cullen and Roy, 2004; Roy and Cullen, 1998). Another population of neurons, termed vestibular-only (VO) neurons, respond much less to active than passive stimulation. Specifically, their activity is reduced by about 60% during active compared to passive vestibular stimulation for those neurons sensitive to translations (Carriot et al., 2013), and by about 70% for rotation-sensitive neurons (Roy and Cullen, 2001), although there is some variability and there are neurons which are less attenuated (Brooks and Cullen, 2014).

The mechanism which causes the suppression of self-generated inputs in the VO neurons is different from a simple subtraction envisaged by von Holst and Mittelstaedt (1950). Unlike in weakly electric fish, where a negative image representing the expected feedback from self-generated activity can be found (Bell, 2001, 1981), no such negative image is present in monkey VO neurons (Roy and Cullen, 2004). The computation underlying the attenuation of self-generated sensory signals must be more complex – it requires a match of actual and predicted proprioceptive feedback from neck proprioceptors (Brooks and Cullen, 2014; Roy and Cullen, 2004). How and where exactly this happens remains to be elucidated, but it is certainly an interesting case, not least because it does not comply with the somewhat simplistic model of von Holst and Mittelstaedt (1950).

5.1.5.1.3 Reliability of vestibular reafference vs. corollary discharge

As the picture of CD becomes more complex with more examples from different behaviours and different animal models, one might wonder how reliable the CD is compared to sensory feedback. For instance, it has been shown for speech efference copy (EC) in humans that EC represents a sensory goal rather than an accurate prediction, since the EC does not contain information about variability in the effectors (Niziolek et al., 2013). For locomotion, the speed affects the predictability of its sensory consequences: Variability in head movements

relative to the mean across locomotor cycles is smaller for running than for walking in humans (MacNeilage and Glasauer, 2017). These authors have speculated that vestibular reafference should therefore be more highly weighted during walking than running, and that the opposite should be true for CD. That reafference is less important at higher locomotor speeds is also supported by the finding that patients with acute unilateral failure have less trouble running than walking (Brandt et al., 1999). Similarly, galvanic vestibular stimulation leads to larger deviations from a straight path during walking than during running in healthy human subjects (Jahn et al., 2000), with similar effects in the visual modality (Jahn et al., 2001). Apparently there is a general trend of higher reliance on centrally generated, stereotyped patterns at higher locomotor speeds, and a higher reliance on sensory feedback at lower locomotor speeds. The mechanisms of how the processing of vestibular inputs changes with active vs. passive movements in monkeys described above might well be different from the mechanisms that deal with reafference during the more stereotypical and rhythmic locomotion. The level of suppression of reafference likely depends on the function – e.g. gaze vs. posture stabilisation – and on the phase of the locomotor cycle (e.g. Bent et al., 2004).

5.1.6 Conclusion: Corollary discharges and reafferent signals

The monkey vestibular system deals with reafference in a way that reflects the function of each sensory processing stream: Vestibular neurons involved in vestibulospinal reflexes are suppressed during head or body movements, since the reflex would likely counteract the voluntary movement (Brooks and Cullen, 2014; Carriot et al., 2013; Roy and Cullen, 2001). The activity of vestibular neurons that are the central part of the three-neuron VOR arc, on the other hand, are not suppressed during head movements, but are affected by gaze efference copy (Roy and Cullen, 2004). Different sensory processing streams are therefore differentially affected by CD, reflecting their functions. This is also evident in how mormyrid fish selectively deal with reafference in their three electrosensory subsystems (Bell, 1989). Both of these examples have CD components that are plastic, and this plasticity relies on the cerebellum, or on cerebellar-like structures (Brooks et al., 2015; Requarth and Sawtell, 2014). More examples of differential as well as plastic effects of CD are likely to be described in the future, as more animals and different behaviours are examined.

CD effects on sensory processing are not necessarily suppressive, or always reduce the sensory sensitivity. Some suppression might selectively enhance sensory coding, for instance in the auditory cortex of marmoset monkeys, where 75% of the neurons are suppressed during vocalisation, but this suppression increases the sensitivity to feedback disturbances (Eliades and Wang, 2008). Moreover, gains in neurons that are involved in visual motion processing are larger during walking than when stationary in the fruit fly (Chiappe et al., 2010). Similarly, responses of neurons in the primary visual cortex of the mouse to visual stimuli are about twice as large when running than when sitting still (Niell and Stryker,

2010), and encoding of visual stimuli is similarly enhanced during locomotion (Dadarlat and Stryker, 2017). However, some of these effects might be due to a more global behavioural state change rather than a specific CD. Discriminating more global behavioural state effects from at least somewhat specific CD effects will be important (e.g. arousal and locomotion have distinct effects on mouse visual cortex (Vinck et al., 2015)), though the boundaries are likely blurred – after all, a CD signal might cause the change in behavioural state.

CD might also contribute to high-level cognitive functions. For instance, the sense of self might rely on CD as well as on sensation – I feel 'myself' because I do just as much as because I feel (Tsakiris et al., 2005). Disorders of auditory CD have been implicated in schizophrenia (Feinberg and Guazzelli, 1999; Ford and Mathalon, 2005). Besides, CD likely is the reason that humans are unable to tickle themselves (Blakemore et al., 1998, 1999).

CD therefore has already been shown to have a plethora of functions: It can suppress as well as enhance sensory inputs, cause motions to facilitate locomotion or even contribute to a sense of self. In addition to this range of adaptive adjustments already uncovered, more details and mechanisms are likely to be found as researchers consider different animals that carry out voluntary movements or locomotion. Eventually, we would like to understand complex interactions between movements and sensation of freely moving animals in their natural habitat. Quantifying reafference as in the kinematics study (chapter 3, Hänzi and Straka, 2017b) or uncovering unusual CD effects as in the study on tentacle retraction (chapter 4, Hänzi et al., 2015) are only the first steps in such an endeavour.

5.2 Locomotor behaviour and navigational strategies

The previous section of the discussion examined locomotion and its consequences – the circuits enabling an animal to locomote, the consequences that this poses both on motor and sensory levels, and the strategies such as CD that are then used to deal with these consequences. The following section, on the other hand, will examine locomotor activity more generally (distinction of locomotion vs. locomotor activity from Martin (2003)), looking at the animal's movement in space and speculating about strategies and functions of particular patterns of movement.

5.2.1 Locomotor behaviour in concave environments

Locomotor behaviour has many facets that are difficult to characterise all at once (Martin, 2003). A very simple locomotor activity assay is the open field (OF) test, where animals are placed in a barren arena and observed for a certain amount of time. Many analyses rely on the x-y coordinates of the animal over time, and assess proximity to the wall, the only physical landmark. In such a simplified and spatially concave environment, a variety of animals has been observed to follow walls, including many species of rodents (Webster et al., 1979;

Wilson et al., 1976), a number of fish (Clements et al., 2002; Kato et al., 1996; Peitsaro et al., 2003; Warren and Callaghan, 1975), larvae of *Aedes* and *Anopheles* (Gonzalez et al., 2017), as well as adult fruit flies (Besson and Martin, 2005; Goetz and Biesinger, 1985). To this list we can now add large tadpoles and young froglets of *X. laevis* (chapter 2, Hänzi and Straka, 2017a). However, without further experiments, wall following (WF) in a concave environment does not imply a specific underlying mechanism (i.e. Tinbergen's causation) or associated function (Tinbergen's survival value). Potential mechanisms and strategies will therefore be discussed below, in the context of further experiments and comparisons.

5.2.2 Interpretations of wall following in the open field

In the literature, two potential functions of WF have been put forward repeatedly: First, the wall as a safe harbour, implying a defensive function, and second, the wall as a help to navigate, implying a spatial function. Both thigmotaxis and centrophobism have been suggested as mechanisms underlying WF. A number of these aspects will now be discussed, often with reference to either of these potential mechanisms or functions. The functions will also be discussed separately in more detail.

5.2.2.1 Effects of differences in the level of illumination

Changing the illumination from bright cold-light LEDs to mostly infrared did not change the WF behaviour of *Xenopus* tadpoles or froglets (chapter 2, Hänzi and Straka, 2017a). This implies that vision or rather its absence is unlikely to be a main driver for WF. This argument is strengthened by finding no difference between the tadpoles and the froglets, since their reliance on vision is very different – froglets react to a visual stimulus being waved above the tank, whereas tadpoles do not. However, other animals increase their WF in the dark, and for these, there are two possible, again not mutually exclusive functions of that behaviour: First, vision is not available in the dark. Animals might therefore follow the wall in the dark to gain information about their environment using some near-range senses such as touch or the lateral line. Indeed, some sighted morphs of Mexican blind cave fish only follow walls in the dark, but not in the light (Sharma et al., 2009), and blindfolded humans employ touch to learn about their environment (Kallai et al., 2007; Yaski et al., 2009). The mechanism underlying WF in this context might well be thigmotaxis. Second, the open space might appear dangerous in the light, implying a defensive function for WF; the associated mechanism might be centrophobism. For instance common spiny mice, which are strictly nocturnal, venture more often into the centre of an OF in the dark or if there are objects which might provide shelter (Eilam, 2004). Similarly, in the field, more nocturnal desert rodents are captured in traps placed in the open if it is darker (Price et al., 1984), and wild-caught prairie deer mice move more along the wall in bright nights (Brillahart and Kaufman, 1991).

In tadpoles of *X. laevis*, neither of these influences of light play a role, since they do not change their WF behaviour with changes in illumination (chapter 2, Hänzi and Straka, 2017a). However, in animals that do show a difference, both strategies and mechanism – acting defensively in the light with centrophobism, or gaining information in the dark with thigmotaxis – might be at play.

5.2.2.2 Changes in wall following with development

After the tadpoles of *X. laevis* hatch (stage 37/38), they prefer to attach themselves to a substrate and rest, even though they are capable of swimming (Boothby and Roberts, 1992; Jamieson and Roberts, 2000). Hatchlings also appear to follow walls less than somewhat older tadpoles (see example trajectories in Currie et al., 2016), although the propensity to swim and the distance covered very likely are confounding factors in this case. After the onset of feeding (stage 45/46), the propensity to swim increases (Currie et al., 2016), and already, many of the animals follow the walls, though not all, and they still frequently cross the central areas of the tank. As the tadpoles grow, they become stronger wall followers, and froglets consistently follow the walls, hardly ever crossing the centre of the tank (chapter 2, Hänzi and Straka, 2017a). WF therefore persists across metamorphosis and its associated change in locomotor style. The reason why smaller tadpoles are weaker wall followers is still unclear – one possibility is that because their axis of rotation of the head movements during swimming is outside the head (Lambert et al., 2009), they can more easily turn away from a wall. Modelling constraints in turning angle in fact increased WF in a concave environment (Creed and Miller, 1990).

Another aspect of the development of *X. laevis* tadpoles is their tentacles, which they transiently possess at large tadpole stages. I originally hypothesised that the animals would specifically use these mechanosensory tentacles (Fig. 8) for thigmotaxis, and therefore expected WF to be strongest at those stages that possess tentacles. However, this was not the case – WF is already present before the tentacles protrude from the face, and it is still strong after the tentacles degenerate. Moreover, animals that for unknown reasons did not develop tentacles were equally strong wall followers (chapter 2, Hänzi and Straka, 2017a). The presence of tentacles therefore is no prerequisite for WF, and while they most likely touch the wall with their tentacles when they have them, they might use their facial skin if they do not.

To my knowledge, no other study has examined WF over a similarly long period of development. In zebrafish, the amount of time the animals spend in the outer half of the tank is high and similar from 3 to 7 dpf (Colwill and Creton, 2011), though this is not a very stringent criterion for WF. Adult zebrafish also follow walls (Anichtchik et al., 2004; Peitsaro et al., 2003), but no study has tested whether this wall following is active (see section 5.2.2.4), which would help to assess the function of WF for these animals.

5.2.2.3 Effects of differences in the size of the environment

In tadpoles of *X. laevis*, I found that animals are stronger wall followers in smaller tanks (chapter 2, Hänzi and Straka, 2017a). This contrasts with the situation in social voles, which generally are strong wall followers, but are even stronger in larger arenas (Eilam, 2003; Eilam et al., 2003). The authors have argued that because there is a difference in the strength of WF between arenas of different sizes, thigmotaxis cannot be the main driving force, since the wall would be equally attractive to touch no matter the size of the environment (Eilam et al., 2003). Even though the effect is in the opposite direction in *X. laevis*, the same argument applies – thigmotaxis cannot be the main driving force behind WF, although to a certain extent this mixes up two of Tinbergen's levels of describing behaviour: Causation/mechanism and survival value/function. Still, this does not imply that either of these animals do not touch the walls when following the walls; it just says that thigmotaxis is unlikely to be the driving force behind the observed WF behaviour. With tadpoles being stronger wall followers in smaller tanks, the idea surfaced that the behaviour is largely constrained by the environment rather than an active choice of the animal. This was confirmed by testing the animals in convex tanks (see below).

5.2.2.4 Active vs. passive wall following

By changing the shape of the environment from concave – such as the common square or circular arenas – to convex, one can judge whether the animals actively follow the wall, or 'only' passively (Creed and Miller, 1990). At a convex curve, the animal can swim straight and leave the wall, or make a turn to actively follow the wall. The tadpoles and froglets of *X. laevis* all mostly swam straight, leaving the wall. Their WF in the square tank can therefore be considered passive, mostly barrier-driven (Creed and Miller, 1990). Such convex environments have only been used in a few other animals. Adult fruit flies mostly walk straight (Soibam et al., 2012), but not quite as consistently as the tadpoles. In contrast to the tadpoles, the flies can also walk on the walls themselves, in which case they would naturally follow them. Flies apparently prefer the boundaries of their environment rather than vertical walls (Soibam et al., 2012), though a comparison to other animals is difficult as hardly anyone uses arenas as sophisticated in their spatial layout as these authors. Convex arenas at least have been used both for cockroaches and blind cave fish. Cockroaches often run with their ipsilateral antenna touching the wall (Camhi and Johnson, 1999), but leave the wall at a convex curve in about 50% of the trials (Creed and Miller, 1990), although the proportion of leaving depends at least somewhat on the radius of the convex curve – if it is too small, the animals always leave. A similar dependence on the radius has been found in blind cave fish (Patton et al., 2010), but if the radius is large enough, the animals turn to follow the wall. Their WF is therefore active, and I would predict that any WF whose main function is to gain information about the environment – which the blind cave fish do using their lateral line

system – will be active. While I have not examined tadpoles and froglets swimming in convex tanks with different radii of the convex curvature, I would predict that changing the radius would only have a small effect, since the animals left the wall at the convex point even when swimming slowly.

The fact that *X. laevis* follows the wall passively suggests that their WF primarily is barrier-driven, and determined to a large extent by the shape and size of the environment. This in turn implies that their WF is unlikely to have a specific navigational or defensive function; rather, it seems to be a by-product of the constraints of the environment.

5.2.2.5 Wall following as a defensive behaviour

WF in rodents has been strongly associated with anxiety (Simon et al., 1994; Treit and Fundytus, 1988), such that more 'anxious' animals are stronger wall followers. While it might be unclear or doubtful whether an animal is 'anxious' the way humans are, the strength of WF changes with the administration of anxiolytic or anxiogenic agents (e.g. Choleris et al., 2001; Gentsch et al., 1987; see Prut and Belzung, 2003). Perhaps the animals have reasons to be 'anxious', since the open space can be dangerous, especially in the presence of a predator (Bonsignore et al., 2008). The fact that WF increases in aversive situations (Grossen and Kelley, 1972) also suggests that WF is a defensive strategy. Indeed, some authors have argued that all rodent OF behaviour is driven by the need for safety, and not exploration – if rats are provided with a shelter in an otherwise barren open field, they hardly ever leave the refuge (Genaro and Schmidek, 2000). Other authors have also argued that rats behave in a way to optimise security (Whishaw et al., 2006). However, voles voluntarily leave a shelter in an OF (Eilam, 2010), showing that security is not the only factor. There is probably a trade-off between optimising security while allowing some exploration. Where along this trade-off an animal ends up likely depends on several factors such as the species, the environment, and its feeding state.

While I have not specifically tested the relationship between WF and anxiety in *X. laevis*, I would predict that any effects of drugs on this behaviour would be small, since their WF appears to be mainly driven by the constraints of the environment (see sections 5.2.2.3 and 5.2.2.4). However, it is also possible that a small effect could be found, since even in walking fruit flies, where the apparent WF results from a preference for boundaries (Soibam et al., 2012), drugs affect WF in a way to suggest it might have an anxiety-related component (Mohammad et al., 2016).

5.2.2.6 Wall following for spatial learning

Some experiments have suggested that WF can help for learning the spatial layout of the environment. For instance, blindfolded humans initially go along the wall (Kallai et al., 2007;

Yaski et al., 2009) before crossing into the centre more often. They then slow down and/or use their arms as they approach a wall in the expectation of hitting something (Yaski et al., 2009). Similarly, mole rats, which explore an OF very slowly in the beginning with many backtrackings such that it takes them several minutes to go once along the perimeter, after a while take brief shortcuts across a corner (Avni et al., 2008). Tadpoles of *X. laevis*, on the other hand, repeatedly swim into the wall head first, suggesting they have not learnt about the boundaries of their environment (Fig. 9).

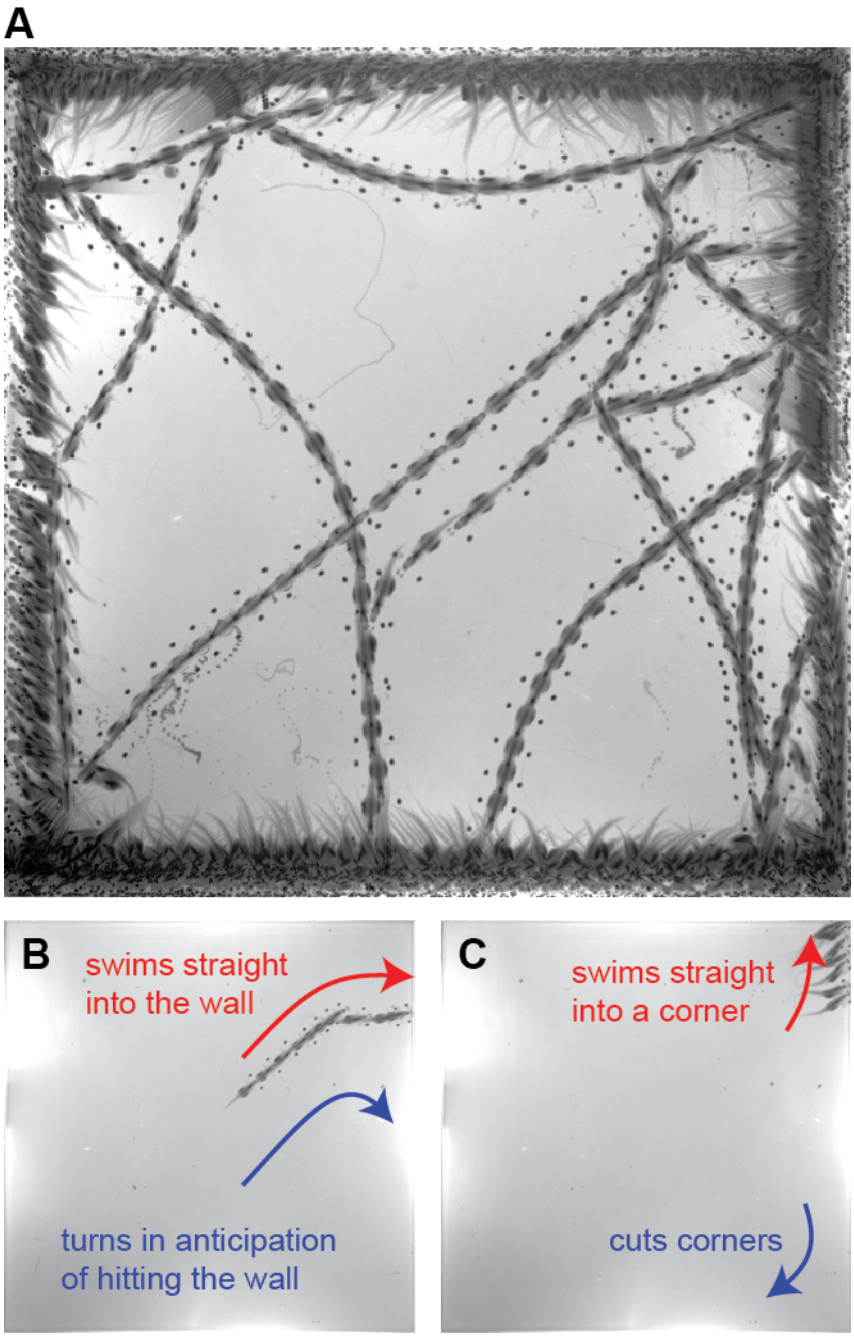

Figure 9. Tadpoles swim straight into walls and are unlikely to have learnt the layout of the environment. A) Overlay of a stage 56 tadpole swimming for 10 min in a shallow tank, at 3 frames per second. Note that the animal often orients itself at 90° to the wall (the tail is towards the centre), as if it was trying to swim into the wall. B, C) Excerpts from A from the last 6 s of the 10 min swimming period. Red describes what the tadpole does, and blue describes what a spatial learner would do. B) The tadpole swims straight into the wall (red), whereas a spatial learner would turn in anticipation of hitting the wall (blue), and therefore avoid a head-on collision. C) The tadpole swims into the corner (red), whereas a spatial learner after a while would have learnt to cut corners (blue).

From readouts of activity levels, it seems that both blindfolded crayfish, which explore their environment with their tactile antennae, as well as blind cave fish, which rely on the lateral line system, learn the layout of their environment (Basil and Sandeman, 2000; Teyke, 1989). I would predict that no such learning would occur in tadpoles even if they were allowed to explore an environment over hours as in the crayfish study.

Interestingly, WF using a near-range sense such as touch (i.e. thigmotaxis) is only useful for spatial learning in the initial stages of exploration. Blindfolded humans often thigmotactically go around the room once, and then start crossing into the centre (Yaski et al., 2009). However, if this thigmotactic behaviour persists, spatial learning suffers (Kallai et al., 2007). Thigmotaxis therefore seems to be useful in an initial stage of exploration, which is also seen in mice (Benjamini et al., 2011; Fonio et al., 2009).

5.2.3 Conclusion: Wall following in the open field

Even such a seemingly simple behaviour such as WF can have complex underpinnings, making the interpretation of WF difficult, especially if WF was only observed in concave environments. The need for safety and the urge to explore the environment might both 'drive' the animal to follow walls in its environment. These potential uses of WF as well as the potential mechanisms underlying WF are not mutually exclusive. As usual in biology, the correct answer in most cases likely is 'a bit of both', and 'it depends on the context'. Moreover, the fact that in some cases, WF can simply be a response to the shape and size of the environment such as in passive WF, or 'pseudothigmotaxis' (Creed and Miller, 1990), highlights the constraints imposed by a spatially simple environment such as the OF. Inadvertently, the experimenter can even influence the animal's behaviour by choosing a circular rather than a square shape – mouse trajectories differ in square and circular arenas even when the animals are fairly far from the wall (Horev et al., 2007). The small square space usually employed for OF studies constrains the animal's behaviour (Benjamini et al., 2010; Cheng, 2005). This again shows that behaviour is difficult to interpret and very careful controls are needed to avoid assigning a function to the animal's behaviour when it really is only responding to the unnatural laboratory environment.

5.3 Conclusion

In this thesis, I have examined the behaviour and some of its neural underpinnings in tadpoles and froglets of *Xenopus laevis*. Examining their movements in a simple concave environment showed that they follow the walls, but that his behaviour is largely driven by the shape and the size of the environment, and is unlikely to serve a defensive or navigational function. While I am a strong supporter of studying behaviour, these experiments have taught me that interpreting behaviour is difficult and very easily influenced even by the best-intending experimenter. Moving on from locomotor behaviour to CPG-driven locomotion, I found that

the swimming frequency of the tadpoles decreases as they grow. Moreover, their left-right head movements, which are yoked to the movements of the tail during swimming, are orders of magnitude larger than what vestibular researchers use in their experiments. This certainly put the stimuli commonly used in vestibular studies into perspective, and reinforced the point that movements come with sensory consequences. Finally, I described an uncommon consequence of locomotion, namely appendage movement driven by a locomotor CD. Taken together, these studies contribute to an improved description of behaviour of a widely used laboratory animal, at different levels of description and detail.

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Appendix

I. Abbreviations

CD	corollary discharge
CPG	central pattern generator
dpf	days post fertilisation
EC	efference copy
hpf	hours post fertilisation
OF	open field
Re	Reynolds number
SCC	semicircular canal
VN	vestibular nucleus
VO	vestibular-only
VOR	vestibulo-ocular reflex

II. Summary in simple words

Since you, dear reader, have made it all the way through my dissertation and are even diligent enough to read the appendix, you deserve a treat. I was inspired by the webcomic xkcd (www.xkcd.com), whose author wrote a comic to describe how a rocket works but limited himself to only the 1000 most common English words (up-goer five, https://xkcd.com/1133/). He then wrote a whole book in this style, explaining complicated things like datacenters, tectonic plates, or cells in simple words (he called them computer buildings, the flat rocks we live on, or the little bags of water you're made of; see https://xkcd.com/thing-explainer/). Raising up to the up-goer five challenge, where scientists have tried to explain what they do in only these restricted terms, I wrote the following summary of my thesis:

How do animals go about their lives? I have tried to answer a number of questions that are a bit like this one, but smaller – no one can study all animals. Instead, I studied the young ones of those animals that we think of as green and jumping around. These young ones are a bit like the ones that always live in water, as they also move about in water. I have wondered why the animals often go along the walls and edges rather than moving about in the open. It turns out that this is probably not something special, but instead the animals do it because in the wild they do not have edges and walls around them. Also, I have tried to figure out how exactly they move - how much do they move their heads, how fast can they get from one place to another? When they move forward in water, they move their heads from left to right very fast! And as they grow from tiny to pretty large, this becomes a bit slower.

Finally, as the animals move, they – by moving themselves – change some other things about their bodies, like what they sense about the world. They can sense when their heads move – so when they move their own head, they can sense that too. Not only what they sense can change though, but also some other things can be moved at the same time as moving forward. One thing that seems to happen in the young animals of a certain age is that they stick out something like fingers when they do not move or only move very slowly. When they move fast, they pull these fingers back such that they are out of the way for moving forward.

So I have studied how these animals move in several ways: One was about how the animals move about and why they move the way they do. Another way asked how exactly they move, like how much do they move their heads, and how this changes as they grow. The third way looked at what happens at the same time as moving but has nothing to do with moving forward.

III. List of publications

Haenzi, S., Stefanics, G., Lanaras, T., Calcagni, M., and Ghosh, A. (2014). Altered cortical activation from the hand after facial botulinum toxin treatment. Ann. Clin. Transl. Neurol. *1*, 64–68.

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Hänzi, S., and Ghosh, A. (2014). Tactile underrepresentation of the forehead along the vertical axis. Clin. Neurophysiol. *125*, 856–858.

Hänzi, S., Banchi, R., Straka, H., and Chagnaud, B.P. (2015). Locomotor corollary activation of trigeminal motoneurons: coupling of discrete motor behaviors. J. Exp. Biol. *218*, 1748–1758.

Hänzi, S., and Straka, H. (2017). Developmental changes in head movement kinematics during swimming in *Xenopus laevis* tadpoles. J. Exp. Biol. *220*, 227–236.

IV. Affidavit ('Eidesstattliche Erklärung')

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation **‚Behaviour and its consequences: *Xenopus laevis* wall following, swimming, and corollary discharge'** selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation **‚Behaviour and its consequences: *Xenopus laevis* wall following, swimming, and corollary discharge'** is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

_____ _____

München, den (Munich, date) Unterschrift (Signature)

V. Author contributions

Developmental changes in head movement kinematics during swimming in *Xenopus laevis* tadpoles

Sara Hänzi and Hans Straka

Wall following in *Xenopus laevis* is passive

Sara Hänzi and Hans Straka

For both manuscripts, SH contributed the following:

Investigation, Software, Visualization: S.H.; Supervision, Project administration, Funding acquisition: H.S.; Conceptualization, Writing: S.H. and H.S.

- Conducting all experiments.
- Analysis all experiments, including writing analysis scripts in Matlab/Python.
- Visualising the results of the experiments.
- Writing, revising the manuscript, together HS; submitting the manuscript.

Date, place:

Signature Sara Hänzi:

Signature Prof. Dr. Hans Straka (Supervisor):

Locomotor corollary activation of trigeminal motor neurons: coupling of discrete motor behaviors

Sara Hänzi, Roberto Banchi, Hans Straka and Boris P. Chagnaud

S.H. and R.B. contributed equally to the present study.

Contributions of SH

- Conducting all behavioral and electrophysiological experiments.
- Analysis of the behavioral part of the study (Fig. 1).
- Analysis for the electrophysiological parts of the study (Fig. 4-6, except duration analysis).
- Writing, revising the manuscript, together with all other authors; submitting the manuscript.

Contributions of RB

- Preliminary experiments and design of the study, together with BPC.
- All experiments and analyses required for the anatomical part of the study including the visualization of rhombomeric domains (Fig. 2).
- Back-filling method for use in calcium imaging.
- All experiments and analyses involved in the calcium imaging of tentacle motoneurons during fictive swimming (Fig. 3).
- Analysis of the duration of the tentacle nerve discharge compared to the duration of fictive swimming (Fig. 5A, B).
- Writing and revising, together with all other authors.

Date, place:

Signature Sara Hänzi:

Signature Roberto Banchi:

Signature Prof. Dr. Hans Straka (Supervisor):