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Research Article: Methods/New Tools

Sensory and Motor Systems

Whole-Body Imaging of Neural and Muscle Activity during Behavior in *Hydra vulgaris*: Effect of Osmolarity on Contraction Bursts

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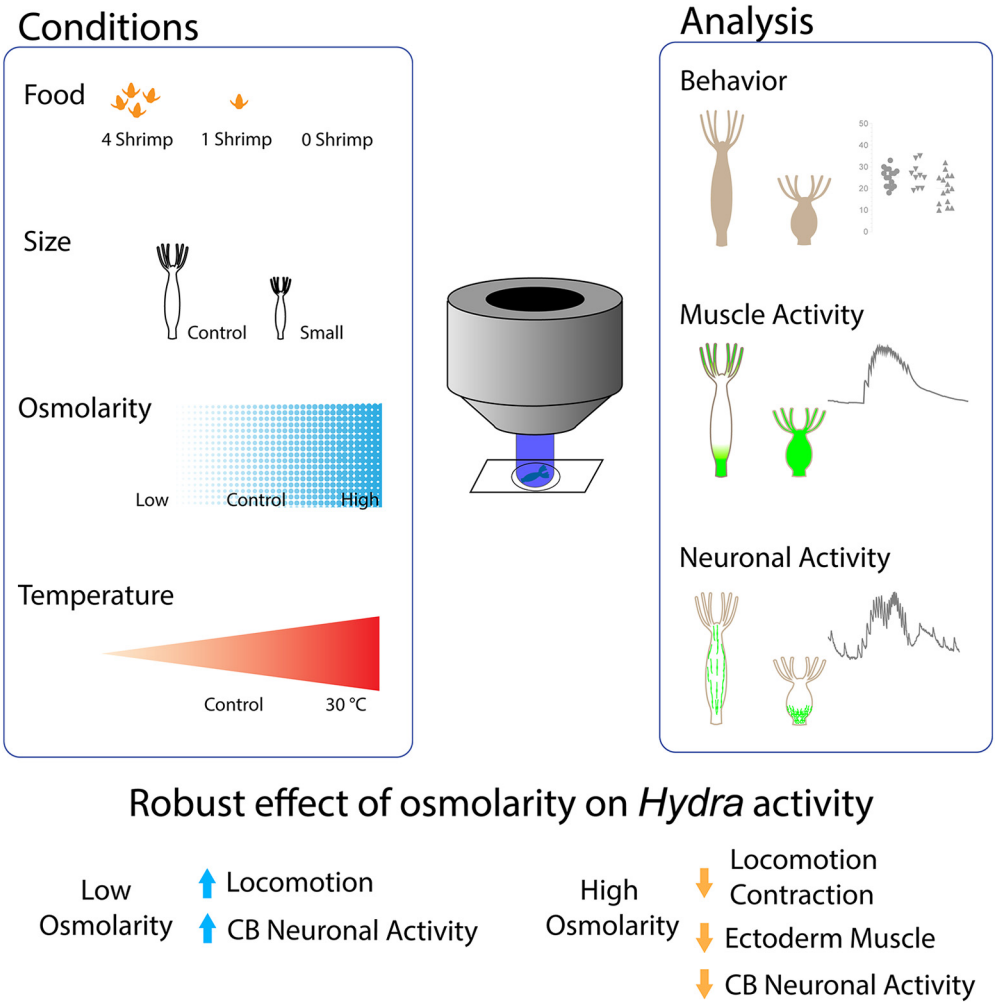
Visual Abstract



Significance Statement

We imaged whole-body muscle and neuronal activity in *Hydra* in response to different physiological and environmental conditions. Osmolarity bidirectionally altered *Hydra* contractile behavior in a reflexive fashion. These changes were accompanied by specific changes in the activity of one neuronal circuit and one set of muscles. By providing neurobiological mechanisms for a reflex in a cnidarian, this work is a step toward comprehensive deciphering of the mechanisms of animal behavior by measuring the activity of all neurons and muscle cells.

The neural code relates the activity of the nervous system to the activity of the muscles to the generation of behavior. To decipher it, it would be ideal to comprehensively measure the activity of the entire nervous system and musculature in a behaving animal. As a step in this direction, we used the cnidarian *Hydra vulgaris* to explore how physiological and environmental conditions alter simple contractile behavior and its accompanying neural and muscle activity. We used whole-body calcium imaging of neurons and muscle cells and studied the effect of temperature, media osmolarity, nutritional state, and body size on contractile behavior. In mounted *Hydra* preparations, changes in temperature, nutrition state, or body size did not have a major effect on neural or muscle activity, or on contractile behavior. But changes in media osmolarity systematically altered contractile behavior and foot detachments, increasing their frequency in hypo-osmolar media solutions and decreasing it in hyperosmolar media. Similar effects were seen in ectodermal, but not in endodermal muscle. Osmolarity also bidirectionally changed the activity of contraction burst (CB) neurons, but did not affect the network of rhythmic potential (RP) neurons in the ectoderm. These findings show osmolarity-dependent changes in the activity of CB neurons and ectodermal muscle, consistent with the hypothesis that CB neurons respond to media hypo-osmolarity, activating ectodermal muscle to generate CBs. This dedicated reflex could serve as an excretory system to prevent osmotic injury. This work demonstrates the feasibility of studying an entire neuronal and muscle activity in a behaving animal.

Introduction

Calcium imaging of neuronal circuits (Yuste and Katz, 1991) has enabled recent investigations of the circuit basis of animal behavior in a number of transparent organisms such as *Caenorhabditis elegans*, *Drosophila* larvae, and zebrafish embryos (Nagel et al., 2005; Liewald et al., 2008; Honjo et al., 2012; Cong et al., 2017; Kim et al., 2017). While these studies have focused on particular parts of the nervous system, to systematically understand the neural code, i.e., the relation between the activity of a nervous system and behavior, it would be ideal to measure the activity of the entire nervous system and the entire muscular tissue during the entire behavioral repertoire of an animal. This is now possible with the transparent fresh-water cnidarian *Hydra vulgaris*, using transgenic strains that express calcium indicators in every neuron (Dupre and Yuste, 2017) and every muscle cell of the body (Szymanski and Yuste, 2019), and applying machine learning to systematically analyze its behavior (Han et al., 2018). *Hydra* has a simple body consisting of ectoderm and endoderm myoepithelial cells. Muscular processes, myonemes, run longitudinally in the ectoderm and radially in the endoderm. Thus, each myoepithelial layer can have distinct functions in different behaviors, but can also become coactive during sustained contractions (Szymanski and Yuste, 2019).

Hydra has one of the simplest nervous system in evolution, with several hundreds to a few thousand neurons, depending on the size of the animal (Hadzi, 1909; Parker, 1919; Westfall et al., 1991). The simplicity of *Hydra*'s system gives hope that systematic measurements of the neural and muscular activity of behaving *Hydra* could be used to decipher the mechanisms of behavior. *Hydra* neurons are believed to be multifunctional. A sensory neuron with sensory cilia also synapses with epithelial cells as a motor neuron (Westfall, 1973). These neurons are organized in two independent nerve nets, in the ectoderm and endoderm (Dupre and Yuste, 2017). *Hydra*'s nerve nets are distributed throughout the body of the animal, without any cephalization (Epp and Tardent, 1978). Several independent neuronal circuits, interspersed within the nerve nets, are active synchronously in an oscillating manner. The

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main ones named contraction burst (CB) and rhythmic potential (RP)1 circuits, involve independent groups of ectoderm neurons, whereas a third circuit, the RP2 circuit, involves endodermal cells (Dupre and Yuste, 2017). These three circuits are associated with three different motor behaviors: CBs (CB circuit), elongation (RP1), and egestion (RP2; Dupre and Yuste, 2017).

Hydra is a fresh-water animal living in ponds, lakes and streams. Because of this, *Hydra* experiences fluctuations in temperature and osmolarity as well as the amount of food available, which determines its body size. Previous research has described *Hydra* responses to changes in environmental and physiological conditions. Those include decreases in contractions with increased osmolarity (Benos and Prusch, 1973) and after feeding (Grosvenor et al., 1996; Rushforth and Hofman, 1972) and necrosis after acute increases in temperature (Bosch et al., 1988). These past studies show that external modification of *Hydra* behavior is possible.

Motivated by this work, we explored systematically how different environmental conditions affect *Hydra* behavior, focusing on body contractions. Do do so, we performed measurements of *Hydra* behavior under standard conditions in mounted and freely behaving animals and used calcium imaging to measure how neurons and muscular cells responds to physiological and environmental conditions important for their survival. Experimental conditions included high or low osmolarity (control, 50 mM sucrose or diH_2O), temperature (23°C or 30°C), food (zero, one, and four shrimp per day for a week), and body size (mature vs newly released buds). In each of these conditions, we measured the number of contractions and foot detachments in behavior assays, the ectodermal and endodermal muscle activity, and the activity of the CB and RP1 neuronal circuits.

We expected to see major changes in behavior, neuronal, and muscle activity, as the chosen conditions are essential to *Hydra* survival. But surprisingly, in mounted preparations, we only found robust effects due to osmolarity. Increased osmolarity decreased contractions frequency, consistent with Benos and Prusch (1973), decreased foot detachments and also decreased the activity of CB neurons and ectodermal muscle cells, whereas decreased osmolarity had opposite effects, as a reflex. Our results indicate that *Hydra*'s CB circuit senses osmolarity to control ectodermal muscle and generate contractile behaviors, revealing a specific neuro-muscular reflex that probably evolved for osmoprotection.

Materials and Methods

Materials

Sucrose and sea salt were purchased from Sigma. Brine shrimp, *Artemia nauplii*, were obtained from Brine Shrimp Direct. We used transgenic *Hydra* expressing GCaMP6s in neurons (Dupre and Yuste, 2017) or in ectoderm/endoderm muscle cells (Szymanski and Yuste, 2019).

Hydra culture

Hydra were maintained in media composed of 1.3 mM $CaCl_2$, 0.02 mM $MgCl_2$, 0.03 mM KNO_3, 0.5 mM $NaHCO_3$, and 0.08 mM $MgSO_4$ in an 18°C incubator. *Hydra* were fed brine shrimp three times a week and were starved for 2 d before an experiment.

Environmental or physiological conditions

The following conditions were used. (1) Food: *Hydra* were fed zero, one, or four shrimp every day for a week. *Hydra* were starved for 1 d before an experiment. (2) Size: *Hydra* with large (~1 cm) or small (~0.3 mm) sizes, chosen after bud separation, were fed once. (3) Temperature: room (23°C) or high temperature (30°C). (4) Osmolarity: *Hydra* were imaged in media with low osmolarity (diH_2O, 0 mOsm/l), control medium (control, *Hydra* media, 5 mOsm/l, fresh water is usually between 2 and 8 mOsm/l), or high (50 mM sucrose, 50 mOsm/l) osmolarity.

Calcium imaging

Wide-field calcium imaging of *Hydra* was conducted at 2 Hz using a fluorescence dissecting microscope (Leica M165) equipped with a long-pass GFP filter set (Leica filter set ET GFP M205FA/M165FC), 1.63× Plan Apo objective, and a sCMOS camera (Hamamatsu ORCA-Flash 4.0). A mercury arc lamp was used to illuminate the sample. *Hydra* were mounted between coverslips with 100- to 200-μm spacers, depending on animal thickness. All imaging was conducted at a room temperature ~23°C unless indicated.

Behavior analysis

The number of contractions and foot detachments were manually scored from calcium imaging movies (mounted *Hydra* between coverslips) or movies of freely moving *Hydra* in glass-bottom dishes (MatTek). Five animals were placed per well (depth is 700–750 μm) for 1-h recordings.

Analysis of neural and muscular activity

Values for whole-body fluorescent intensity in each frame over time were obtained with ImageJ and used to detect CB and RP1 pulses using a semi-automated program in MATLAB. Whole-body muscle activity was analyzed in the same manner.

Analysis of body column width

Hydra were imaged at 0.5 Hz using a dissecting microscope (Leica M165), 1.63× Plan Apo objective, and sCMOS camera (Hamamatsu ORCA-Flash 4.0). *Hydra* were mounted between coverslips with around 200-μm spacer in control media or in high-osmolarity solution (50 mM sucrose). To measure width, the body column of *Hydra* was fitted into ellipse using a program written by MATLAB. The lowest values from each cycle were used to calculate average width at the end of the elongation.

Statistical methods

Data are shown as average ± SEM in figures and in the text. Two-tailed unpaired Student's *t* test or one-way

Table 1: Statistical tests and results

Figure	Description	Methods	95% CI of difference	Significant	*p* value
1*B*	Food: 0 vs 1	1	–2.355 to 6.718	No	0.4707
	Food: 0 vs 4	1	–3.537 to 5.537	No	0.8506
	Food: 1 vs 4	1	–5.718 to 3.355	No	0.7981
	Osmo: Ctr vs low	1	–6.364 to 4.864	No	0.9432
	Osmo: Ctr vs high	1	0.2450 to 10.25	Yes	0.038
	Osmo: Low vs high	1	0.3148 to 11.69	Yes	0.0367
	Size: Ctr vs small	2	–9.991 to –2.937	No	0.0008
	Temp: Ctr vs high	2	–0.5233 to 6.023	No	0.0958
1*C*	Food: 0 vs 1	1	–2.198 to 1.335	No	0.8207
	Food: 0 vs 4	1	–1.448 to 2.085	No	0.898
	Food: 1 vs 4	1	–0.9775 to 2.478	No	0.5411
	Osmo: Ctr vs low	1	–2.688 to 0.3822	No	0.1728
	Osmo: Ctr vs high	1	1.034 to 3.682	Yes	0.0003
	Osmo: Low vs high	1	1.958 to 5.064	Yes	<0.0001
	Size: Ctr vs small	2	0.08979 to 2.894	Yes	0.0378
	Temp: Ctr vs high	2	–0.9724 to 1.722	No	0.5716
1*E*	Food: 0 vs 1	1	–1.740 to 2.407	No	0.9195
	Food: 0 vs 4	1	0.3931 to 4.540	Yes	0.0164
	Food: 1 vs 4	1	0.05976 to 4.207	Yes	0.0426
	Osmo: Ctr vs low	1	0.7542 to 6.579	No	0.01
	Osmo: Ctr vs high	1	–0.2806 to 4.642	No	0.0925
	Osmo: Low vs high	1	–3.947 to 0.9758	Yes	0.3223
	Size: Ctr vs small	2	4.300 to 21.17	No	0.0059
	Temp: Ctr vs high	2	–6.122 to –2.412	Yes	<0.0001
1*F*	Food: 0 vs 1	1	–1.026 to 0.09217	No	0.1178
	Food: 0 vs 4	1	–1.426 to –0.3078	Yes	0.0014
	Food: 1 vs 4	1	–0.9588 to 0.1588	No	0.2029
	Osmo: Ctr vs low	1	1.610 to 3.724	Yes	<0.0001
	Osmo: Ctr vs high	1	0.6877 to 2.474	Yes	0.0002
	Osmo: Low vs high	1	–1.979 to –0.1925	Yes	0.0134
	Size: Ctr vs small	2	–0.1413 to 0.9413	No	0.1413
	Temp: Ctr vs high	2	–2.683 to –0.9838	Yes	<0.0001
2*C*	Food: 0 vs 1	1	–176.2 to 327.3	No	0.8033
	Food: 0 vs 4	1	–257.1 to 281.1	No	0.9991
	Food: 1 vs 4	1	–315.3 to 188.2	No	0.8705
	Osmo: Ctr vs low	1	–147.0 to 148.8	No	0.9998
	Osmo: Ctr vs high	1	12.02 to 307.8	Yes	0.0356
	Osmo: Low vs high	1	–6.375 to 324.4	No	0.0588
	Size: Ctr vs small	2	–138.6 to 167.4	No	0.8303
	Temp: Ctr vs high	2	–132.3 to 152.1	No	0.8738
2*D*	Food: 0 vs 1	1	–318.4 to 280.4	No	0.981
	Food: 0 vs 4	1	–473.7 to 125.1	No	0.2655
	Food: 1 vs 4	1	–475.4 to 164.8	No	0.378
	Osmo: Ctr vs low	1	–174.6 to 237.8	No	0.9107
	Osmo: Ctr vs high	1	–106.8 to 282.0	No	0.4681
	Osmo: Low vs high	1	–150.2 to 262.2	No	0.7494
	Size: Ctr vs small	2	2.523 to 332.7	Yes	0.0473
	Temp: Ctr vs high	2	–20.35 to 199.1	No	0.0955
2*E*	Food: 0 vs 1	1	–13.68 to 17.47	No	0.939
	Food: 0 vs 4	1	–19.72 to 15.75	No	0.948
	Food: 1 vs 4	1	–20.83 to 13.08	No	0.8034
	Osmo: Ctr vs low	1	–19.22 to 0.7527	No	0.0686
	Osmo: Ctr vs high	1	–6.431 to 13.54	No	0.5872
	Osmo: Low vs high	1	1.625 to 23.96	Yes	0.0273
	Size: Ctr vs small	2	–7.207 to 13.24	No	0.5081
	Temp: Ctr vs high	2	–4.729 to 13.86	No	0.2836

(Continued)

Table 1: Continued

Figure	Description	Methods	95% CI of difference	Significant	*p* value
2F	Food: 0 vs 1	1	–19.97 to 13.83	No	0.9455
	Food: 0 vs 4	1	–30.51 to 3.289	No	0.1296
	Food: 1 vs 4	1	–28.61 to 7.526	No	0.3429
	Osmo: Ctr vs low	1	–18.81 to 7.069	No	0.4634
	Osmo: Ctr vs high	1	–7.909 to 16.49	No	0.6216
	Osmo: Low vs high	1	–2.777 to 23.11	No	0.1307
	Size: Ctr vs small	2	2.745 to 22.78	Yes	0.0188
	Temp: Ctr vs high	2	0.5891 to 18.07	Yes	0.0396
2G	Food: 0 vs 1	1	–1.613 to 1.854	No	0.9773
	Food: 0 vs 4	1	–0.8072 to 2.899	No	0.2839
	Food: 1 vs 4	1	–0.8077 to 2.659	No	0.3176
	Osmo: Ctr vs low	1	0.5862 to 3.721	Yes	0.0108
	Osmo: Ctr vs high	1	1.379 to 4.514	Yes	0.0017
	Osmo: Low vs high	1	–0.9592 to 2.545	No	0.4373
	Size: Ctr vs small	2	–7.207 to 13.24	No	0.5081
	Temp: Ctr vs high	2	–4.729 to 13.86	Yes	0.2836
2H	Food: 0 vs 1	1	–2.115 to 3.010	No	0.8669
	Food: 0 vs 4	1	–2.517 to 2.607	No	0.9985
	Food: 1 vs 4	1	–3.142 to 2.336	No	0.9032
	Osmo: Ctr vs low	1	–0.4909 to 5.189	No	0.1103
	Osmo: Ctr vs high	1	–2.518 to 2.609	No	0.9988
	Osmo: Low vs high	1	–5.037 to 0.4289	No	0.1028
	Size: Ctr vs small	2	–2.405 to 1.561	No	0.6416
	Temp: Ctr vs high	2	–2.067 to 1.073	No	0.4785
3C	Food: 0 vs 1	1	–168.3 to 323.3	No	0.6736
	Food: 0 vs 4	1	–276.1 to 190.3	No	0.8709
	Food: 1 vs 4	1	–353.6 to 112.8	No	0.3699
	Osmo: Ctr vs low	1	–448.7 to –9.334	Yes	0.0406
	Osmo: Ctr vs high	1	5.853 to 341.4	Yes	0.0422
	Osmo: Low vs high	1	192.3 to 612.9	Yes	0.0005
	Size: Ctr vs small	2	–96.12 to 287.7	No	0.288
	Temp: Ctr vs high	2	–173.1 to 196.4	No	0.8855
3D	Food: 0 vs 1	1	–890.9 to 180.7	No	0.2575
	Food: 0 vs 4	1	–594.9 to 429.8	No	0.9638
	Food: 1 vs 4	1	–198.1 to 743.2	No	0.3624
	Osmo: Ctr vs low	1	–398.8 to 148.8	No	0.4752
	Osmo: Ctr vs high	1	–285.7 to 221.3	No	0.9411
	Osmo: Low vs high	1	–160.7 to 346.3	No	0.6139
	Size: Ctr vs small	2	–189.6 to 358.8	No	0.497
	Temp: Ctr vs high	2	–430.9 to 270.0	No	0.5946
3E	Food: 0 vs 1	1	–20.65 to 14.51	No	0.8669
	Food: 0 vs 4	1	–31.19 to 3.966	No	0.1246
	Food: 1 vs 4	1	–29.33 to 8.249	No	0.2875
	Osmo: Ctr vs low	1	–18.11 to 16.66	No	0.9932
	Osmo: Ctr vs high	1	–9.921 to 18.47	No	0.7082
	Osmo: Low vs high	1	–12.39 to 22.38	No	0.7294
	Size: Ctr vs small	2	–4.836 to 10.90	No	0.406
	Temp: Ctr vs high	2	–4.654 to 17.22	No	0.2095
3F	Food: 0 vs 1	1	–878.5 to 168.3	No	0.1957
	Food: 0 vs 4	1	–583.0 to 417.9	No	0.8911
	Food: 1 vs 4	1	–187.2 to 732.3	No	0.2734
	Osmo: Ctr vs low	1	–23.83 to 22.78	No	0.998
	Osmo: Ctr vs high	1	–25.81 to 12.84	No	0.6477
	Osmo: Low vs high	1	–28.53 to 16.61	No	0.7608
	Size: Ctr vs small	2	–6.952 to 12.37	No	0.536
	Temp: Ctr vs high	2	–4.654 to 17.22	No	0.2095

(Continued)

Table 1: Continued

Figure	Description	Methods	95% CI of difference	Significant	*p* value
3*G*	Food: 0 vs 1	1	–0.6621 to 3.852	No	0.1787
	Food: 0 vs 4	1	–1.813 to 2.470	No	0.908
	Food: 1 vs 4	1	–3.408 to 0.8747	No	0.2816
	Osmo: Ctr vs low	1	–6.687 to –1.087	Yes	0.0066
	Osmo: Ctr vs high	1	–0.5830 to 4.411	No	0.1499
	Osmo: Low vs high	1	3.165 to 8.437	Yes	<0.0001
	Size: Ctr vs small	2	–1.356 to 3.915	No	0.3006
	Temp: Ctr vs high	2	–0.8085 to 4.736	No	0.1378
3*H*	Food: 0 vs 1	1	–9.974 to 2.307	No	0.2484
	Food: 0 vs 4	1	–6.661 to 4.990	No	0.919
	Food: 1 vs 4	1	–2.828 to 8.823	No	0.3722
	Osmo: Ctr vs low	1	–389.9 to 139.9	No	0.4301
	Osmo: Ctr vs high	1	–382.1 to 229.7	No	0.7785
	Osmo: Low vs high	1	–257.1 to 354.7	No	0.901
	Size: Ctr vs small	2	–3.063 to 4.773	No	0.6283
	Temp: Ctr vs high	2	–6.933 to 3.432	No	0.4402
4*D*	High vs Ctr	2	5.575 to 43.27	Yes	0.0193

Method 1 indicates ordinary one-way ANOVA, Tukey's multiple comparison test, and method 2 indicates unpaired *t* test. The four conditions used were food (Food), osmolarity (Osmo), size (Size), and temperature (Temp). Control medium (ctr).

ANOVA with Tukey's multiple comparison test were conducted in GraphPad Prism software (Table 1).

Code accessibility

All code is available as Extended Data 1. The MATLAB code was used to analyze neural and muscular activity in Figs. 2–4.

Results

Hydra's contractile behavior affected by media osmolarity

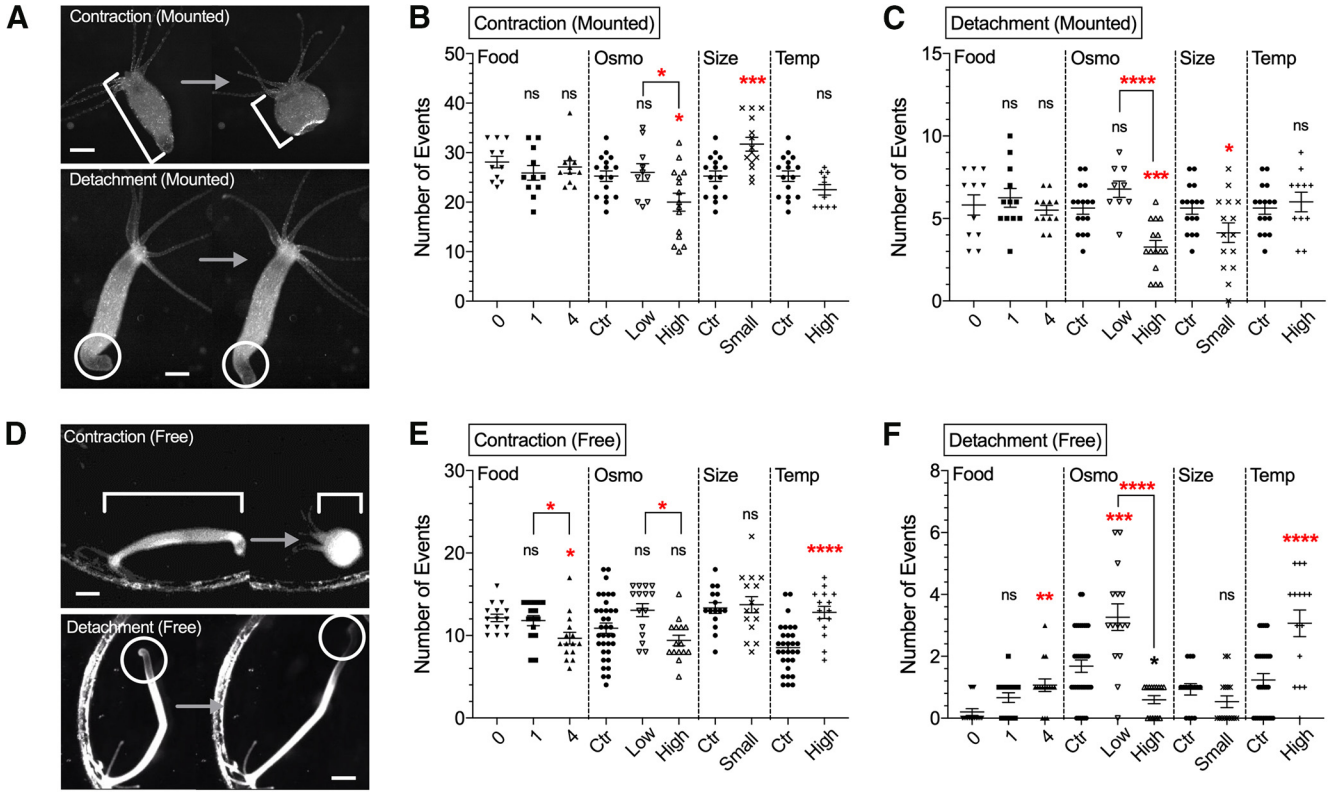
Hydra has a small repertoire of highly stereotypical behaviors (Han et al., 2018). One of the most noticeable ones are spontaneous periodic contractions, known as "contraction bursts" (Wagner, 1905; Reis and Pierro, 1955; Passano and Mccullough, 1964). Possible roles of contractions by *Hydra* include foraging, protection by retraction (Miglietta et al., 2000; Swain et al., 2015), food digestion (Shimizu and Fujisawa, 2003), and excreting excess water from the body (Macklin et al., 1973). Another common behavior of *Hydra* is locomotion, i.e., translocation of the foot from one place to another. This is initiated by "foot detachment," where the basal disk detaches from a substrate's surface (Rodrigues et al., 2016).

We first tested how these two simple behaviors of *Hydra* were affected by various physiological and environmental conditions. Conditions chosen included amount of food, osmolarity or temperature of media, and the size of an animal. For the amount of food, *Hydra* was starved for 1 d before an experiment. For each condition, the frequency and duration of contractions and foot detachments were measured. In mounted preparations, where specimens are place in a microscope chamber with a spacer, osmolarity or body size robustly changed the frequency of contractions (Fig. 1*A–C*; see Materials and Methods). High-osmolarity media significantly decreased the frequency of contractions compared with control (Fig. 1*B*, $p = 0.0380$) or low-osmolarity conditions (Fig. 1*B*, $p = 0.0367$). Similarly, high-osmolarity media significantly decreased the number of foot detachments compared with control (Fig. 1*C*, $p = 0.0003$) or low-osmolarity conditions (Fig. 1*C*, $p < 0.0001$). Also, smaller size *Hydra* had more contractions (Fig. 1*B*, $p = 0.0008$) but fewer foot detachments (Fig. 1*C*, $p = 0.0378$).

As mounting restricts *Hydra* behavior, because of compression of body between glass coverslips, we also imaged freely moving *Hydra* under widefield illumination in the same conditions (Movie 1). Consistent with results in mounted preparations (Fig. 1*B*,*C*), in free moving animals, high osmolarity also decreased the number of contractions compared with low osmolarity (Fig. 1*E*, $p = 0.0100$) and the number of foot detachments, compared with control (Fig. 1*F*, $p = 0.0134$) or low-osmolarity conditions (Fig. 1*F*, $p < 0.0001$). But, unlike mounted preparations, well-fed (four shrimp per day) *Hydra* did not show any difference in behavior, comparing with control conditions. (Fig. 1*B*, $p = 0.8506$ for contractions; Fig. 1*C*, $p = 0.8980$ for detachments). Also, in well-fed freely moving *Hydra*, the number of contractions decreased (Fig. 1*E*, $p = 0.0164$), while the number of foot detachments increased (Fig. 1*F*, $p = 0.0014$). High temperature also increased contractions (Fig. 1*E*, $p < 0.0001$) and foot detachments (Fig. 1*F*, $p < 0.0001$) in freely moving animals. Overall, osmolarity was the only parameter that robustly changed behavior in both freely moving and mounted specimens. As motor behaviors must be generated as a result of contractile force derived from muscle, we next assessed how these changes in behaviors are accounted for the activity of muscle cells. For these experiments, we used exclusively mounted preparation, as it is yet not feasible to image and reconstruct the activity of neurons and muscle cells in freely moving animals.

Bidirectional effects of osmolarity on ectodermal muscle activity

Hydra's body is composed of two layers of cells: ectodermal and endodermal epitheliomuscular tissues. Both



epithelia are separated by an extracellular matrix called mesoglea. Inside these epithelial layers, there is a gastrovascular cavity that functions as a both gut and vasculature and carries nutrients to the entire body (Shimizu and Fujisawa, 2003). Both ectoderm and endoderm epitheliomuscular tissues generate action potentials (Dupre and Yuste, 2017; Szymanski and Yuste, 2019), which likely propagate through gap junctions (Westfall et al., 1980). These muscle cells contract in a calcium-dependent manner through myonemes, intracellular muscle processes that run longitudinally along the ectoderm and radially in