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RESEARCH ARTICLE

Plasticity in fluctuating hydrodynamic conditions: tube foot regeneration in sea urchins

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ABSTRACT

Regenerating structures critical for survival provide excellent model systems for the study of phenotypic plasticity. These body components must regenerate their morphology and functionality quickly while subjected to different environmental stressors. Sea urchins live in high-energy environments where hydrodynamic conditions pose significant challenges. Adhesive tube feet provide secure attachment to the substratum but can be amputated by predation and hydrodynamic forces. Tube feet display functional and morphological plasticity in response to environmental conditions, but regeneration to their pre-amputation status has not been achieved under quiescent laboratory settings. In this study, we assessed the effect of turbulent water movement, periodic emersion and quiescent conditions on the regeneration process of tube foot morphology (length, disc area) and functionality (maximum disc tenacity, stem breaking force). Disc area showed significant plasticity in response to the treatments; when exposed to emersion and turbulent water movement, disc area was larger than that of tube feet regenerated in quiescent conditions. However, no treatment stimulated regeneration to pre-amputation sizes. Tube foot length was unaffected by treatments and remained shorter than non-amputated tube feet. Stem breaking force for amputated and non-amputated treatments increased in all cases when compared with pre-amputation values. Maximum tenacity (force per unit area) was similar among tube feet subjected to simulated field conditions and amputation treatments. Our results suggest a role of active plasticity of tube foot functional morphology in response to field-like conditions and demonstrate the plastic response of invertebrates to laboratory conditions.

KEY WORDS: *Strongylocentrotus purpuratus*, Reaction norm, Echinoderm adhesion, Tensile breaking force, Tenacity

INTRODUCTION

Phenotypic plasticity is broadly defined as a single genotype expressing multiple phenotypes (DeWitt and Scheiner, 2004). For morphological traits, phenotypic plasticity is a process that occurs throughout ontogeny (DeWitt and Scheiner, 2004) with significant ecological and evolutionary consequences (Forsman, 2015; Pigliucci, 2001). Central to understanding these consequences is whether plasticity is active or passive (Forsman, 2015). Active plasticity strongly suggests adaptation (Forsman, 2015; Whitman and Agrawal, 2009), where environmental conditions act as a signal to an organism to change the phenotypic expression of a trait (e.g. chemical cues for inducible defenses). Conversely, passive plasticity is less likely to be adaptive; instead, the environment acts directly on a trait and its expression is often the result of an organism's susceptibility to the environment (e.g. smaller size as a result of low-quality diet) (Forsman, 2015; Scheiner, 2006; Whitman and Agrawal, 2009). Both active and passive processes may play a role in the expression of a single trait (Whitman and Agrawal, 2009).

Understanding phenotypic plasticity in response to environmental conditions is important in habitats with extreme fluctuations in physical conditions, such as the intertidal and shallow subtidal zones of wave-swept open coasts (Denny, 1988; Denny and Gaylord, 1996; Jensen and Denny, 2015). In these habitats, hydrodynamic conditions fluctuate annually, seasonally and daily with tidal cycles, currents and turbulent waves. These hydrodynamic regimes subject organisms to drag and lift forces, posing significant challenges for secure attachment to the substratum (Denny, 1988; Jensen and Denny, 2015; Santos and Flammang, 2005; Siddon and Witman, 2003). Additionally, tide cycles can leave organisms in the intertidal zone emersed (i.e. exposed to air), causing aerobic and metabolic distress (Capparelli et al., 2021; Defur, 1988; McGaw et al., 2015). Indeed, prolonged exposure to air during low tide raises body temperature and leads to desiccation (Allen et al., 2012), threatening survival. These repeated, and often unpredictable, stressors are important drivers of community function and structure of shallow coastal environments (Denny, 1985; Paine and Levin, 1981).

Sea urchins are key members of benthic communities in the intertidal and subtidal zone; their high density and powerful feeding apparatus (Aristotle's lantern) enable them to modify the habitat by consuming large amounts of macroalgae (Steneck, 2020) and bioeroding rock substrates (Davidson and Grupe, 2015; Russell et al., 2018). One crucial adaptation allowing sea urchins to survive and feed in these challenging habitats is the ability to firmly attach to heterogeneous substrates (Flammang, 1996; Santos et al., 2005; Stark et al., 2020) and catch drift algae (Rodriguez, 2003) using adhesive tube feet. These structures occur along the oral (bottom, in direct contact with substrate)–aboral (top, extended in the water column) axis and have different functions depending on their location. Oral tube feet are used mostly for adhesion to the substrate and aboral tube feet are used for catching drift algae, respiration and sensing (Leddy and Johnson, 2000; Lesser et al., 2011). Tube feet are projections of the water vascular system and consist of an extendable stalk with an adhesive disc at the distal end (Santos et al., 2013; Smith, 1978). The discs adhere to the substrate using a duo-gland system, secreting an adhesive to attach and a de-adhesive to detach (Flammang, 1996). Some discs, however, remain glued to the substrate when sea urchins are pulled by hydrodynamic forces or

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predators, causing stem material failure and tube foot amputation (Narvaez et al., 2020; Santos and Flammang, 2005; Smith, 1978). Like other echinoderms, sea urchins can regenerate soft and hard structures, including tube feet (Bodnar and Coffman, 2016; Brown and Caldwell, 2017; Loram and Bodnar, 2012; Reinardy et al., 2015). The impact of environmental conditions associated with a tidal cycle, such as wave action and emersion, on the regeneration process of tube feet may influence their ability to attach and feed, ultimately affecting survival.

Sea urchins exhibit high phenotypic plasticity that can provide them with a variety of morphological (Ebert, 1996), physiological (Adams et al., 2011) and behavioral adaptations (Harding and Scheibling, 2015) to thrive in challenging environmental conditions. Plasticity occurs across their ontogeny (Ebert, 2020; McAlister and Miner, 2018; Siikavuopio et al., 2012), in numerous body components such as the gonads (Christiansen and Siikavuoplo, 2007; Russell, 1998), skeleton (Byrne and Hernandez, 2020; Hernandez and Russell, 2010; Selden et al., 2009), jaw (deVries et al., 2019; Ebert, 1980) and tube feet (Narvaez et al., 2020; Stark et al., 2020); and over short periods of time (Cohen-Rengifo et al., 2018; Santos and Flammang, 2007; Toubarro et al., 2016). Their ability to regenerate body components makes them excellent candidates to study this phenomenon, as the plastic response to environmental conditions can be stimulated by amputating body parts in the laboratory and evaluating the regeneration process under the presence/absence or varying intensities of different environmental conditions.

Tube feet display a high level of phenotypic plasticity in response to environmental conditions (Fenner, 1973; Leddy and Johnson, 2000; Narvaez et al., 2020; Santos and Flammang, 2005, 2006, 2007, 2008; Stark et al., 2020). Sea urchins respond to wave action by increasing the number of tube feet attached to the substrate and by increasing the extensibility and toughness of the tube foot stem (Cohen-Rengifo et al., 2017, 2018, 2019; Santos and Flammang, 2007). Likewise, tube foot disc surface area and amputation rate vary in response to native substrate (i.e. rock type; Narvaez et al., 2020), and total adhesive capability increases with temperature (Santos and Flammang, 2007; Wilbur and Moran, 2018). Thus, the attachment capacity of a sea urchin is influenced by the interaction of environmental conditions with features such as tube foot morphology, adhesive secretion, the number of tube feet adhering to the substrate and amputation/regeneration rate (Cohen-Rengifo et al., 2017; Narvaez et al., 2020; Santos and Flammang, 2007, 2008; Sharp and Gray, 1962; Smith, 1978; Stark et al., 2020).

Phenotypic plasticity is difficult to observe in the field (DeWitt and Scheiner, 2004; Gianoli and Valladares, 2012), particularly in exposed rocky intertidal and shallow subtidal habitats (Miner et al., 2005). Several studies on sea urchin phenotypic plasticity have been conducted in laboratory settings (*ex situ*) under controlled experimental conditions (Crook and Davoren, 2016; deVries et al., 2019; Ebert, 1996; Hernandez and Russell, 2010; McAlister and Miner, 2018; Narvaez et al., 2020; Russell et al., 2018; Scholnick and Winslow, 2020; Selden et al., 2009). Simulating tidal cycles in the laboratory requires reproducing high hydrodynamic forces and long periods of emersion. Indeed, maximum water speed in rocky intertidal zones ranges from 5 to 25 m s^{-1} depending on shoreline topography and wave height (Denny, 1985; Gaylord, 1999; Jones and Demetropoulos, 1968).

A recent study on the purple sea urchin *Strongylocentrotus purpuratus* showed the lack of challenging environmental conditions associated with quiescent laboratory conditions (gentle water movement and constant immersion) resulted in the reduction of tube foot disc size and adhesive capability (Narvaez et al., 2020) in less than 200 days. The same study showed that even after 6 months, regenerating tube feet did not recover their initial length, disc area or adhesive capability in quiescent laboratory settings. Similarly, in *Paracentrotus lividus*, the protein responsible for adhesion decreased its expression significantly when sea urchins were transported to the laboratory (Toubarro et al., 2016) and sea urchin total adhesive force decreased when they were removed from the field (Cohen-Rengifo et al., 2018; Santos and Flammang, 2007). These studies demonstrate a clear plastic response of tube feet, both regenerating and undamaged, and suggest that a lack of environmentally relevant stressors results in reduced performance of tube feet.

Understanding the plastic response of body components that are critical for survival is particularly important in organisms that are susceptible to the multiple stressors associated with climate change. Marine organisms must cope with the progressive increase in mean water temperature and decreases in pH (Harley et al., 2006). However, they must also quickly acclimate to acute stressors that are predicted to increase in intensity and frequency, such as marine heatwaves (Oliver et al., 2018), storms (Kossin et al., 2020) and hyposalinity events (Cheng et al., 2020). The ability of sea urchin tube feet to display phenotypic plasticity on short temporal scales provides an ideal system to study the adaptive advantage that plasticity confers to individuals under challenging environmental conditions.

Here, we examined the plasticity and regenerative properties of *S. purpuratus* tube feet exposed to hydrodynamic conditions of a simulated tidal cycle regime. Using controlled laboratory conditions that simulate the water turbulence and emersion of a wave-swept intertidal zone, we predicted that tube foot morphology (length and disc area) and functionality (maximum disc tenacity and stem tensile breaking force) of regenerating tube feet would show a plastic response driven by environmental conditions. Specifically, we expected regenerating tube feet would recover to the functional performance levels measured pre-amputation when exposed to high water turbulence and emersion, but not when exposed to quiescent conditions.

MATERIALS AND METHODS

Sea urchin collection and maintenance

The Aquatic Resources Group Service (University of California, Davis) collected shallow, subtidal (6–8 m depth) sea urchins, *Strongylocentrotus purpuratus* (Stimpson 1857), near Bodega Bay, CA, USA, on 11 October 2019. The collection site was a 'barren ground' – high density of sea urchins and no kelp (Lawrence, 1975). Shipment to Villanova University was delayed until 25 October 2019, because of California wildfires (the Kincade Fire). Before transport, sea urchins were kept inside collection bags hanging in a tank with running seawater to avoid damage to the tube feet. Immediately upon arrival at Villanova University, sea urchins were placed in a 1000 l recirculating water system and acclimated to laboratory conditions for 24–48 h before any manipulations. The experimental water system had a single sea table (182.9×91.4×16.5 cm length×width×height) with an adjustable standpipe to control depth (Russell et al., 2018). Sea urchins were fed rehydrated kelp (Wel Pac) *ad libitum*, and the system and sea urchin cages (see below) were cleaned daily. Water temperature (mean±s.d.: 11.4±0.5°C, n=109) and salinity (mean±s.d.: 32.3 ±0.6‰, n=109) were monitored daily, and water chemistry (Ca, Mg, pH, dKH, P, NH_3) was monitored twice per week and remained stable over the course of the experiment.

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Experimental design

To assess the effect hydrodynamic conditions have on the regeneration process of tube feet, we subjected sea urchins to three treatments: turbulent water, periodic emersion and quiescence (see below). To ensure even distribution of sizes across treatments, we separated sea urchins into four groups (n=11–15 per group) so mean size (test diameter) and variance were approximately equal ($F_{3,46}$=0.044, P=0.988; Table S1). One group provided initial measurements (n=11) of tube foot morphology and functionality in which tube feet were not amputated (i.e. initial sea urchins), and the other three were randomly assigned to the three experimental treatments in which one ambulacral column of tube feet was amputated (i.e. experimental sea urchins).

Three days after the sea urchins arrived in the laboratory, we simulated a catastrophic loss of tube feet in all three experimental groups (i.e. turbulent water, periodic emersion and quiescence) by cutting tube feet and spines from one ambulacral column (adjacent counterclockwise to the madreporite) using nail clippers and a small pair of scissors (Bodnar and Coffman, 2016; Narvaez et al., 2020; Reinardy et al., 2015). The amputated column was re-examined after 24 h and any remaining (non-amputated) tube feet were cut. Spines were cut periodically to provide an unobstructed view of regenerating tube feet. After tube foot amputation, experimental sea urchins were held in cages for 5 days before the start of treatments. Final measurements of tube foot morphology and functionality were performed on all experimental sea urchins 119 days after the start of treatments on both amputated and non-amputated tube feet and the oral and aboral body locations.

Sea urchins in the turbulent and quiescent water treatments were housed in individual cages. Because of limited sea table space, the sea urchins in the periodic emersion treatment were housed together in larger group cages and were excluded from statistical analyses (Fig. 1). Cages were built from segments of PVC pipes (individual cage diameter: 7.7 cm, height: 6 cm; group cage: diameter: 20.3 cm, height: 11 cm). Cage bottoms had a cut-out tripod support for a stand, and a plastic mesh (8 mm aperture) secured to the perimeter. Cages stood on the bottom of the sea table over air diffusers that bubbled air up through the mesh bottoms (Fig. 1). Food consumption was quantified twice a week by assessing the change in wet mass of the kelp after 24 h.

Periodic emersion

To simulate the periodic emersion experienced by intertidal sea urchins during low tide, we suspended the emersion cage just above the waterline for 2 h per day. After 14 days, we changed to 1 h per day because four sea urchins died (i.e. 7 of the initial 11 remained). To increase sample size in this treatment, we added a second cage with six sea urchins 4 days after the beginning of the experiment (n=13 for emersion treatment).

Turbulent water

Sea urchins in the turbulent water treatment were exposed to conditions mimicking the combined effect of wave impact (turbulence) and changes in exposure to waves associated with tidal periodicity. Five individual cages were connected to a common PVC pipe that had a submersible pump (2000 l h^{-1} 85 W; Growneer) at each end. The pumps directed a strong jet of water into the pipe and the only exit for the water was through polypropylene adapters (0.85 cm diameter) connecting each cage to the common pipe (Fig. 1). This set-up was repeated 3 times (three pipes, each with five cages, n=15). When the pumps were on, an intense jet of water flowed into each cage at a velocity of 277.97±16.36 cm s^{-1} (mean±s.d., n=150). Water exited the cage through the large mesh opening on the bottom at 3.45±0.19 cm s^{-1}. Thus, depending on the location of the sea urchin within the cage, sea urchins were exposed to multidirectional turbulence inside each cage with a water velocity between ca. 3.45 and 277.00 cm s^{-1}. Water flow was measured weekly by determining the water volume per second exiting the adapter (measured in ml s^{-1}, equivalent to cm^3 s^{-1}). Water speed (v; cm s^{-1}) was estimated with the formula $v=Q/A$, where Q is water flow (cm^3 s^{-1}) and A is the cross-sectional area of the adapter (0.57 cm^2 for entry water velocity) or the cage bottom (46.57 cm^2, exit water velocity). Individual cages were disconnected from the pipes and rotated weekly among the positions of the three pipes to control for the slight variation in water velocity due to position along the pipes and between sets of pumps.

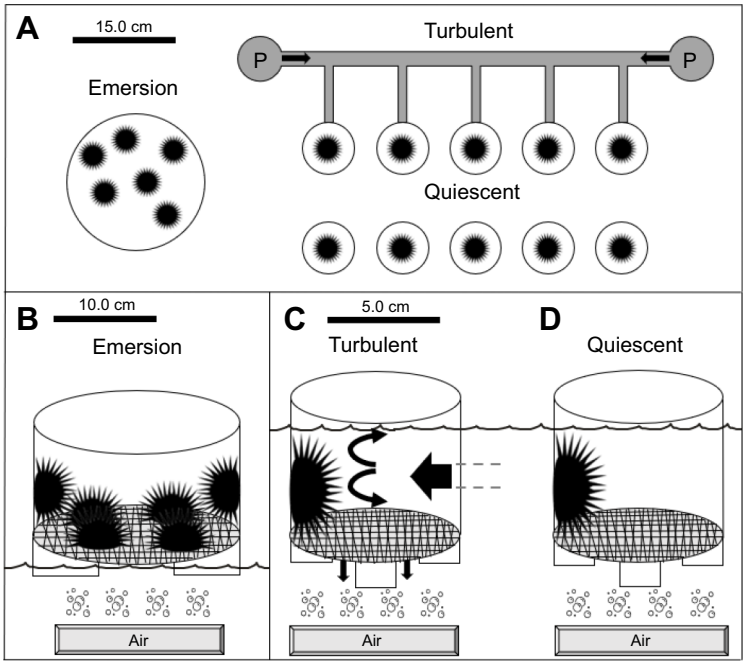

Fig. 1. Schematic diagram of the experimental layout. All sea urchins were maintained in cages constructed of PVC and held in a common sea table (depth 16.5 cm, 182.9×91.4 cm) that shared the same filtration system. Overhead (A) and side views (B–D) of emersion (B), turbulent (C) and quiescent (D) treatments. The emersion cages (n=13, housed in two cages) were elevated above the water line for 2 h day^{-1} for the first 14 days and 1 h day^{-1} for the remaining 105 days (illustrated with water level below mesh). Turbulent (n=15) and Quiescent (n=11) cages housed individual animals that were always immersed (only five cages per treatment are illustrated). To maximize water velocity in the turbulent cages, the outflows of two submersible pumps (P) were set on either end of a common PVC pipe (diameter 1.27 cm). The only outlet for the water was through the small connections (diameter 0.85 cm) to each of 5 turbulent cages. The water velocity into a cage was ∼277±16 cm s^{-1}. The quiescent cages were not connected to pumps and only had air diffusers underneath them. The positions of all the cages were rotated weekly to account for subtle differences in water velocity (turbulent), amount of aeration from diffusers or any other unnoticed/unrecognized variation in the sea table.

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We simulated a modified tide cycle (i.e. 24 h rather than 24 h 50 min) to facilitate water system maintenance. Each cycle was: two high tides (4 h each), two low tides (4 h each), and four changing tides (two rising and receding tides, 2 h each). To simulate turbulence in each tide period, the pumps were connected to a power strip turned on and off by an Arduino electronic board. During the 4 h high tide period, the pumps repeated an on (4 s) and off (8 s) cycle simulating turbulent water movement in a tide pool at high tide. During the 4 h low tide period, the pumps were off, simulating the lack of wave exposure. The 2 h rising tide period was divided into 24, 5 min segments. In each segment, the pumps repeated the on (4 s) and off (8 s) cycle at increasing frequency (1–24 times), simulating increasing exposure to waves in the tide pool on a rising tide. For example, in the first 5 min segment, the pumps were on for 4 s and off for the remaining 292 s. In the second 5 min segment, the pumps were on for 4 s, off for 8 s, on for 4 s and then off for the remaining 280 s. In the last 5 min segment (preceding the high tide period), the pumps repeated the on (4 s) and off (8 s) cycle 24 times (Fig. 2). The 2 h receding tide period mirrored the pattern of the increasing tide period, with the frequency of on/off cycles decreasing (24–1 times) on each 5 min segment (Fig. 2).

Quiescence

Sea urchins in the quiescent treatment were kept at standard laboratory conditions and exposed only to cage aeration (Fig. 1; n=11).

Tube foot morphological and functionality measurements

We measured tube foot morphology (disc surface area and tube foot length) and functionality (maximum disc tenacity and stem tensile breaking force, TBF) of the oral and aboral tube feet in initial and experimental sea urchins. For initial sea urchins, measurements were taken on non-amputated tube feet (no tube feet were amputated on these animals) and for experimental sea urchins, measurements were taken from amputated and non-amputated tube feet. All measurements were taken by immobilizing the sea urchin in a hollowed-out sponge to prevent tube feet from adhering to a solid smooth surface. The target area for measurement was left exposed (i.e. oral or aboral). The sponge with the sea urchin in it was then fitted inside a PVC collar (13 cm diameter) and put in a 4.6 l plastic bucket (18×18×18 cm) filled with 11°C filtered seawater. Regenerating tube feet were considered functional when the disc was able to attach to a substrate and resist tensile force. By the end of the experiment, all regenerating tube feet were longer than spines and able to attach.

Disc surface area and maximum tenacity

Tube foot disc surface area (mm^2) of initial sea urchins was assessed by placing a glass Petri dish on top of the PVC collar containing the submerged, immobilized sea urchin, and allowing the tube feet to attach to the glass. For experimental sea urchins, disc surface area was assessed by placing a rectangular piece of glass (2.5 cm×1.2 cm) near the submerged sea urchin and allowing tube foot attachment. The second method reduces the potential damage to tube foot morphology when removing the glass. With both methods, a 1 mm scale was attached to the side of the glass where the tube foot attached and, once at least 5 tube feet were visibly attached, a photograph was taken with an Olympus Tough TG-6 digital camera (12 MP; see fig. 1 of Narvaez et al., 2020). The mean surface area of the attached tube feet was estimated by measuring the adhesive epidermis and the peripheral disc of each tube foot using ImageJ (Abramoff et al., 2004; Narvaez et al., 2020).

Tube foot maximum disc tenacity (maximum force required to detach a tube foot per unit area, in MPa) was calculated with the formula $T_{max}=(F/A)\times10^{-6}$ where F is the maximum adhesive force (N) required to detach one tube foot and A is the average tube foot disc surface area of the same sea urchin (m^2). Maximum adhesive force was measured by allowing a tube foot to adhere to the side of a capillary tube connected to a 5 N digital force gauge (FGE-XY, Nidec-Shimpo Instruments, Glendale Heights, IL, USA) and then applying a consistent vertical force until the disc detached from the capillary tube (Fig. S1). To ensure uniformity of pulling rate across trials, only one person (C.A.N.) conducted tenacity trials. The highest value of four pulls, conducted on different tube feet, was used to estimate tube foot maximum disc tenacity. We were not able to keep track of individual sea urchins in the emersion treatment, so estimation of maximum disc tenacity for those sea urchins was not possible.

Tube foot length and tensile breaking force (TBF)

Tube foot length (mm) was estimated by allowing sea urchins to extend their tube feet and taking a photograph with a 1 mm scale bar held approximately 2.5 cm below the water. We measured the length of five tube feet (the distance from the base of the tube foot to the base of the distal disc) in ImageJ and calculated mean tube foot length per sea urchin.

TBF was estimated as the maximum force (N) required to cause tube foot stem material failure, regardless of tube foot length or area. A single tube foot was clamped by a metal clip (BlastCase Steel Toothless Alligator Clips, John Miller, Inc.) at approximately half

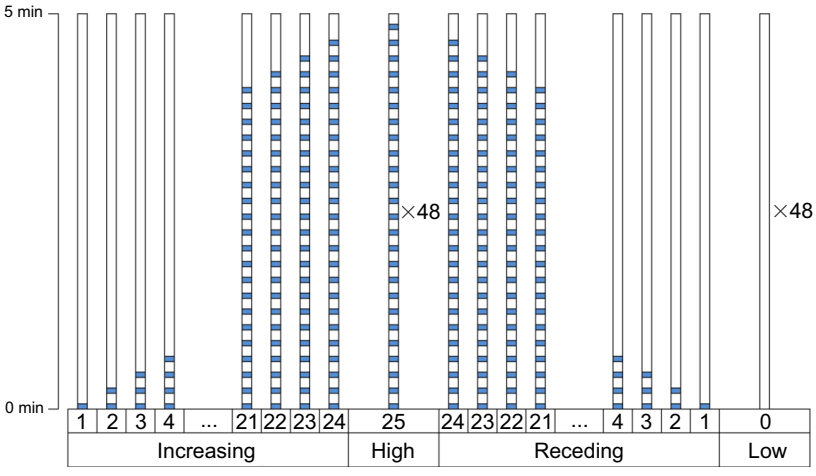

Fig. 2. Visual representation of half (12 h) of the 24 h tide cycle of the turbulent water movement treatment mimicking the hydrodynamics a sea urchin population inhabiting a tide pool would experience. Each vertical bar represents a 5 min segment within each tide period (increasing, high, receding, low). Within each 5 min segment, the blue rectangles represent the time pumps were turned on (4 s), creating a turbulent water flow. White rectangles represent periods when pumps were turned off. During the low tide period (4 h), pumps were off. During the increasing tide period (2 h), pumps were turned on for 4 s at increasing frequency (1–24 times) in each 5 min segment. During the high tide period (4 h), pumps were on for 4 s, 25 times on each 5 min segment (48 times total). During the receding tide period (2 h), pumps were turned on for 4 s at decreasing frequency on each 5 min segment (25–1 times).

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Table 1. ANOVA table of experimental sea urchin tube foot disc area, maximum disc tenacity, length and stem tensile breaking force (TBF)

			Disc area		Tenacity		Length		Stem TBF	
	Numerator d.f.	Denominator d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment	1	24	26.380	**<0.001**	0.305	0.586	0.319	0.577	0.366	0.551
Amputation	1	72	357.584	**<0.001**	0.054	0.818	152.908	**<0.001**	0.002	0.962
Body location	1	72	937.985	**<0.001**	0.084	0.773	8.034	**0.006**	314.690	**<0.001**
Treatment:Amputation	1	72	34.152	**<0.001**	0.081	0.777	2.460	0.121	0.751	0.389
Treatment:Body location	1	72	0.517	0.474	0.093	0.762	0.406	0.526	1.274	0.263
Amputation:Body location	1	72	1.964	0.165	0.047	0.829	2.511	0.117	2.655	0.108
Treatment:Amputation:Body location	1	72	0.124	0.725	0.977	0.326	0.065	0.800	0.841	0.362

Measurements were disc area (mm^2), maximum disc tenacity (MPa), tube foot length (mm) and stem TBF (N). Factors in the model were: hydrodynamic conditions (levels: turbulent, quiescent), amputation (levels: amputated, non-amputated) and body location (levels: oral, aboral). Average tube foot length and disc area were transformed with natural logarithm to meet the normality of residuals assumption. Bold indicates statistically significant differences among factors.

its length and pulled at a constant rate (approximate ~2.54 cm s^{-1}) until breakage. To ensure uniformity of extension rate across trials, only one person (A.Y.S.) conducted TBF trials. The metal clip was connected to a 5 N digital force gauge (FGE-XY, Nidec-Shimpo Instruments, Glendale Heights, IL, USA) with a fishing line (12 lb Shakespeare Omniflex, Shakespeare, Colombia, SC, USA) registering the maximum breaking force (Fig. S1). We calculated the mean TBF of three tube feet per sea urchin.

Statistics

Statistical analyses and graphs were executed in R (https://www.r-project.org/). The emersion treatment was excluded from all the quantitative analyses because of non-independence of measurements taken on sea urchins housed in group cages; however, this group was included in Results and in graphs for comparison. We conducted three separate factorial ANOVA. First, we evaluated tube foot morphology and functionality of experimental sea urchins. This 2×2×2 ANOVA tested the effect of hydrodynamic conditions (levels: turbulent, quiescent), amputation (levels: amputated, non-amputated) and body location (levels: oral, aboral) on tube foot disc area (mm^2), maximum disc tenacity (MPa), length (mm) and stem TBF (N). A second ANOVA compared morphology and functionality of non-amputated tube feet of initial sea urchins with non-amputated tube feet of experimental sea urchins. This 3×2 ANOVA tested the effect of treatment (levels: initial, turbulent, quiescent) and body location (levels: oral, aboral) on tube foot disc area (mm^2), maximum disc tenacity (MPa), length (mm) and stem TBF (N). Finally, we used a third ANOVA to compare morphology and functionality of non-amputated tube feet of initial sea urchins with amputated tube feet of experimental sea urchins. This 3×2 ANOVA tested the effect of treatment (levels: initial, turbulent, quiescent) and body location (levels: oral, aboral) on tube foot disc area (mm^2), maximum disc tenacity (MPa), length (mm) and stem TBF (N). We used a general least square model to test the effect of hydrodynamic conditions (levels: turbulent, quiescent) on sea urchin food consumption during the 17 weeks of the experiment. We included a temporal correlation (AR1) to account for non-independence of the residuals due to the repeated measurements.

Model assumptions were verified graphically. Normal distribution of residuals was assessed with a histogram and homogeneity of variances was verified by plotting the model residuals versus each categorical predictor (Figs S2–S4).

RESULTS

Disc area of experimental sea urchins at the end of the experiment was significantly affected by the interaction between hydrodynamic conditions (turbulent, quiescent) and amputation (amputated, non-amputated; Table 1). Amputated tube feet of experimental sea urchins regenerated smaller discs relative to those of non-amputated tube feet under turbulent and quiescent conditions in both body locations. However, the turbulence treatment stimulated regeneration of oral and aboral tube feet with disc areas 48% and 42% larger, respectively, than those of sea urchins in the quiescent treatment (Fig. 3). Sea urchins in the emersion treatment achieved an intermediate disc size (Fig. 3), with regenerated oral and aboral tube feet having 22% and 25% larger disc areas, respectively, than those of sea urchins in the quiescent treatment (but smaller than those of sea urchins in the turbulent treatment). There was no statistical difference in tube foot disc area of initial sea urchins and non-amputated tube feet of experimental sea urchins (Table 2, Fig. 3). However, disc area of experimental sea urchins in the quiescent treatment showed a tendency to be smaller than that of experimental sea urchins subjected to turbulence and initial sea urchins (Fig. 3). Finally, tube foot disc area of initial sea urchins was larger than the disc area of amputated tube feet measured from experimental sea urchins (Table 3, Fig. 3).

Tube foot maximum disc tenacity of experimental sea urchins did not change as a function of hydrodynamic conditions, amputation, or body location (Table 1, Fig. 4). There was no statistical difference in maximum disc tenacity between initial sea urchins and non-

Table 2. ANOVA table of initial and experimental sea urchin amputated tube foot disc area, maximum disc tenacity, length and stem TBF

			Disc area		Tenacity		Length		Stem TBF	
	Numerator d.f.	Denominator d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment	2	34	3.934	0.049	0.074	0.929	21.080	**<0.001**	0.258	0.774
Body location	1	34	1648.528	**<0.001**	0.157	0.695	231.368	**<0.001**	26.962	**<0.001**
Treatment:Body location	2	34	0.170	0.844	0.476	0.625	3.680	0.036	0.311	0.735

Measurements were disc area (mm^2), maximum disc tenacity (MPa), tube foot length (mm) and stem TBF (N). Factors in the model were: tube feet of sea urchins used to obtain initial values and non-amputated tube feet of experimental sea urchins under the hydrodynamic conditions (levels: turbulent, quiescent, initial) and body location (levels: oral, aboral). Average tube foot length and disc area were transformed with natural logarithm to meet the normality of residuals assumption. Bold indicates statistically significant differences among factors.

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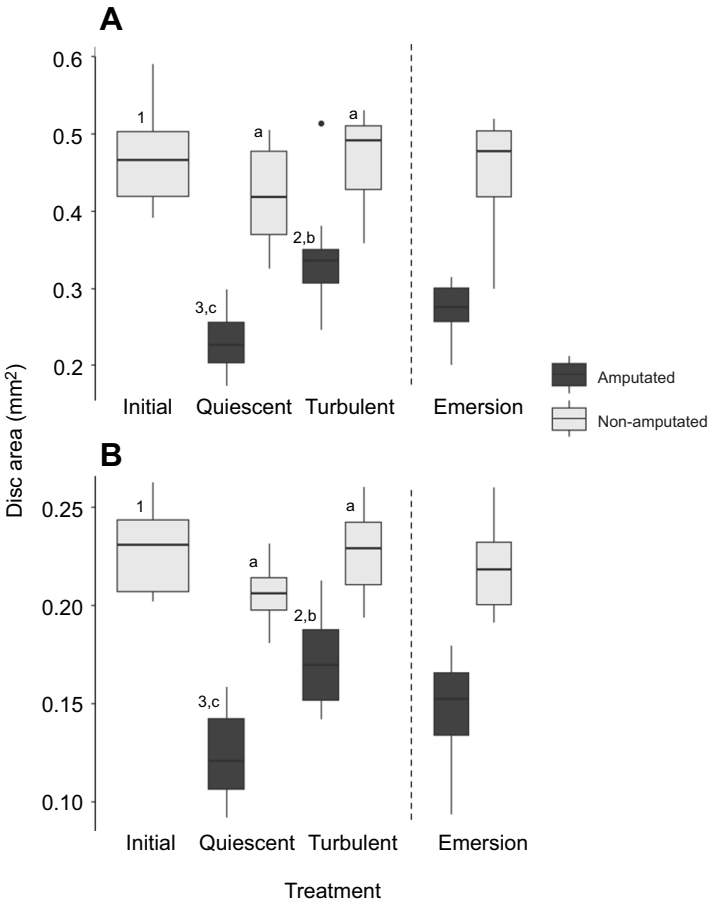

Fig. 3. Disc surface area of initial and experimental sea urchins. Experimental sea urchins were subjected to hydrodynamic conditions (quiescent and turbulent) and amputation (amputated, non-amputated); those in the initial group did not undergo tube foot amputation. Disc area was evaluated on tube feet in the oral (A) and aboral (B) body locations. Statistical analyses excluded the emersion treatment, but measured values are included for reference. The boxplot horizontal line is the median, box edges are the 25th and 75th percentiles; whiskers indicate the largest value within 1.5 times the interquartile range (IQR) and points beyond are values greater than 1.5 times the IQR but less than 3 times the IQR. Tukey *post hoc* test results between amputated and non-amputated tube feet of experimental sea urchins (quiescent versus turbulent) are represented by lowercase letters. Tukey *post hoc* test results between initial and amputated tube feet are represented by numbers. There was no difference in disc area between initial sea urchins and non-amputated experimental sea urchins. Body locations had significantly different disc surface area (oral>aboral).

amputated tube feet of experimental sea urchins (Table 2, Fig. 4), or between initial sea urchins and amputated tube feet of experimental sea urchins (Table 3, Fig. 4).

There was no effect of hydrodynamic conditions on tube foot length of experimental sea urchins (Table 1, Fig. 5). Tube feet were always shorter in amputated than in non-amputated columns and in aboral than in oral body locations (Table 1, Fig. 5). There was no statistical difference between tube foot length of initial sea urchins and non-amputated tube feet of the experimental sea urchins (Table 2, Fig. 5). Amputated tube feet of experimental sea urchins were shorter than those of initial sea urchins. Across treatments, tube feet of experimental sea urchins regenerated to only 54% (oral) and 55% (aboral) of the tube foot length of initial sea urchins (Table 3, Fig. 5).

Stem TBF did not change in response to hydrodynamic conditions or between amputated and non-amputated columns but was significantly higher on the oral than on the aboral side (Table 1, Fig. 6). Stem TBF of non-amputated and amputated tube feet of experimental sea urchins (Tables 2 and 3, respectively) was higher than stem TBF of initial sea urchins (Fig. 6).

Food consumption was not significantly different across experimental treatments ($F_{2,1033}$=0.5449, P=0.5801).

DISCUSSION

The results of our study show that sea urchins exhibit phenotypic plasticity during tube foot regeneration in response to environmental stressors such water turbulence and emersion. Tube foot disc area is particularly plastic and changes in response to water turbulence by increasing in size. Tube foot discs are viscoelastic; they behave elastically under rapidly applied forces (e.g. water turbulence) and distribute stress along the area where tube feet attach to the substrate (Santos et al., 2005). An increase in tube foot attachment area may reduce the risk of detachment by providing more surface area to distribute stress from hydrodynamic forces

Table 3. ANOVA table of initial and experimental sea urchin non-amputated tube foot disc area, maximum disc tenacity, length and stem TBF

			Disc area		Tenacity		Length		Stem TBF	
	Numerator d.f.	Denominator d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment	2	34	67.374	**<0.001**	0.274	0.762	18.110	**<0.001**	15.041	**<0.001**
Body location	1	34	668.828	**<0.001**	**0.013**	0.911	187.814	**<0.001**	5.706	**0.023**
Treatment:Body location	2	34	1.261	0.296	0.113	0.894	2.276	0.118	1.174	0.321

Measurements were disc area (mm²), maximum disc tenacity (MPa), tube foot length (mm) and stem TBF (N). Factors in the model were: tube feet of sea urchins used to obtain initial values and amputated tube feet of experimental sea urchins under the hydrodynamic treatments (levels: turbulent, quiescent, initial) and body location (levels: oral, aboral). Average tube foot length and disc area were transformed with natural logarithm to meet the normality of residuals assumption. Bold indicates statistically significant differences among factors.

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while increasing the amount of adhesive used by each tube foot. Thus, the increased disc area in the turbulent treatment could be a form of active plasticity that would increase the sea urchin's ability to withstand water turbulence and increase survival. However, regenerating tube feet are not functional for some time. We defined a regenerating tube foot as functional when the disc could attach to a substrate (independent of the length of the stem), the stem could resist tensile force, and their length was longer than spines (thus able to reach the substrate). Future studies should determine in which order tube foot functionality is recovered (disc attachment, tensile strength, sufficient length) and when full functionality is regained.

The intermediate disc area achieved by regenerating tube feet in the emersion treatment, although not statistically tested, likely resulted from competing physiological and morphological active plasticity (Kurashige and Callahan, 2007). In the laboratory, sea urchins were often observed attached to the walls of cages when emersed, so tube feet bore the full weight of the sea urchin in the absence of buoyant force. Increased tube foot disc area would represent an active response to decrease the likelihood of dislodgement while emersed, by increasing the amount of adhesive used by each tube foot. It is unclear, however, at which point in the experiment the shorter, regenerating, tube feet were long enough to extend to reach the substrate. Thus, the influence of gravity in the plastic response of regenerating tube feet was likely relevant for only a portion of the experiment. When emersed, sea urchins also faced significant physiological stress (Burnett et al., 2002; Capparelli et al., 2021; McGaw et al., 2015). Thus, sea urchins may invest more energy in the maintenance of homeostasis (active plasticity), resulting in less energy expenditure in the regeneration of tube foot disc area, limiting sea urchin tube foot ability to actively respond to emersion. Differential allocation of resources under stressful conditions is a common response in invertebrates (Glazier and Calow, 1992; Zera and Harshman, 2001), including sea urchins (Guillou et al., 2000; Haag et al., 2016; James and Siikavuopio, 2015; Narvaez et al., 2020). Trade-offs in energy allocation become even more important during the energetically costly regeneration process. In lobsters, molting is delayed when regenerating a limb, and freshwater crabs delay limb regeneration

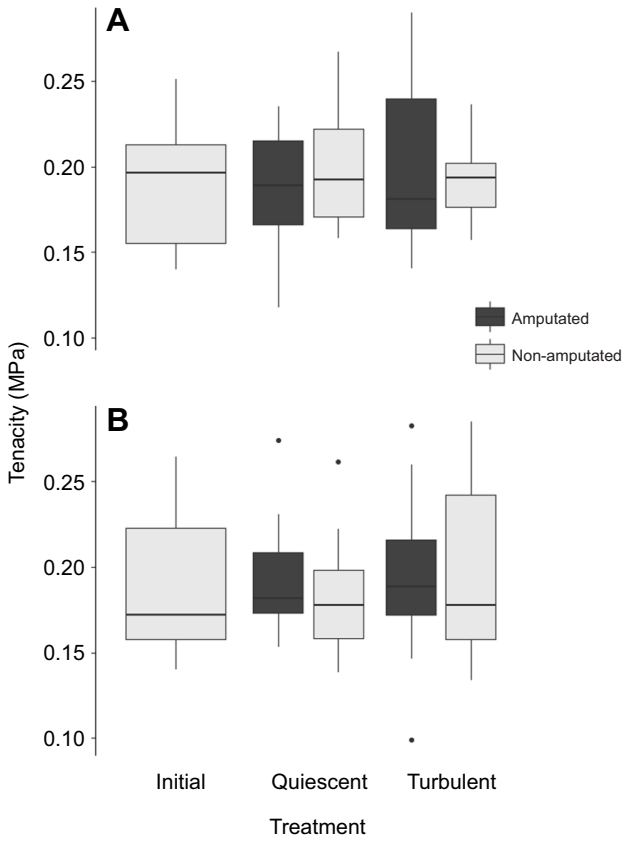

Fig. 4. Maximum disc tenacity of initial and experimental sea urchins. Experimental sea urchins were subjected to hydrodynamic conditions (quiescent and turbulent) and amputation (amputated, non-amputated); those in the initial group did not undergo tube foot amputation. Tenacity was evaluated on discs in the oral (A) and aboral (B) body locations. The boxplot horizontal line is the median, box edges are the 25th and 75th percentiles; whiskers indicate the largest value within 1.5 times the IQR and points beyond are values greater than 1.5 times the IQR but less than 3 times the IQR. There was no difference between treatments, amputation or body locations.

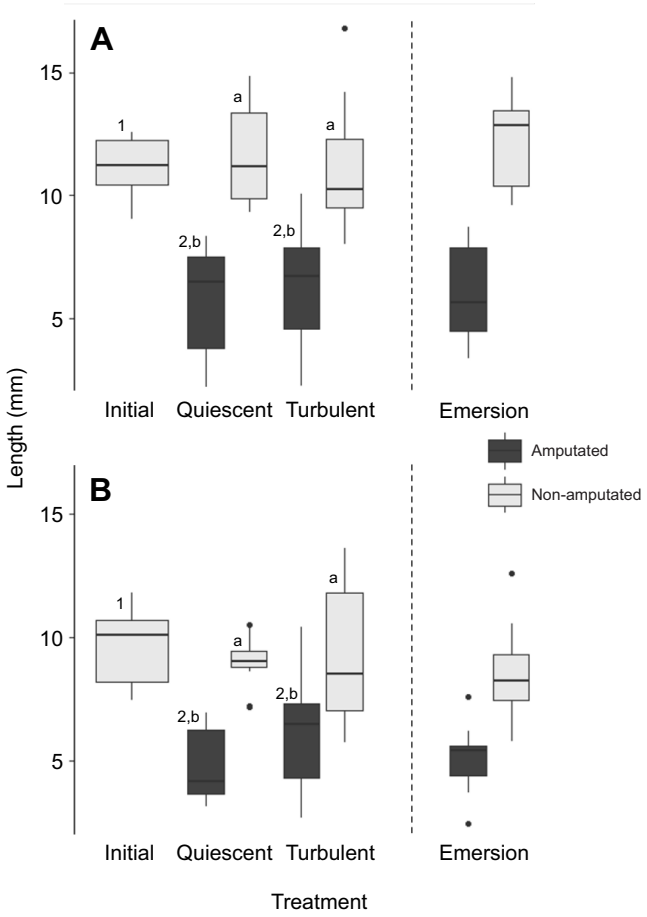

Fig. 5. Tube foot length of initial and experimental sea urchins. Experimental sea urchins were subjected to hydrodynamic conditions (quiescent and turbulent) and amputation (amputated, non-amputated); those in the initial group did not undergo tube foot amputation. Tube foot length was evaluated in sea urchins in the oral (A) and aboral (B) body locations. Statistical analyses excluded the emersion treatment, but measured values are included for reference. The boxplot horizontal line is the median, box edges are the 25th and 75th percentiles; whiskers indicate the largest value within 1.5 times the IQR and points beyond are values greater than 1.5 times the IQR but less than 3 times the IQR. Tukey *post hoc* test results between amputated and non-amputated tube feet of experimental sea urchins (quiescent versus turbulent) are presented with lowercase letters. Tukey *post hoc* test results between initial and amputated tube feet of experimental sea urchins are presented with numbers. There was no difference between initial sea urchins and non-amputated tube feet from experimental sea urchins or among body locations.

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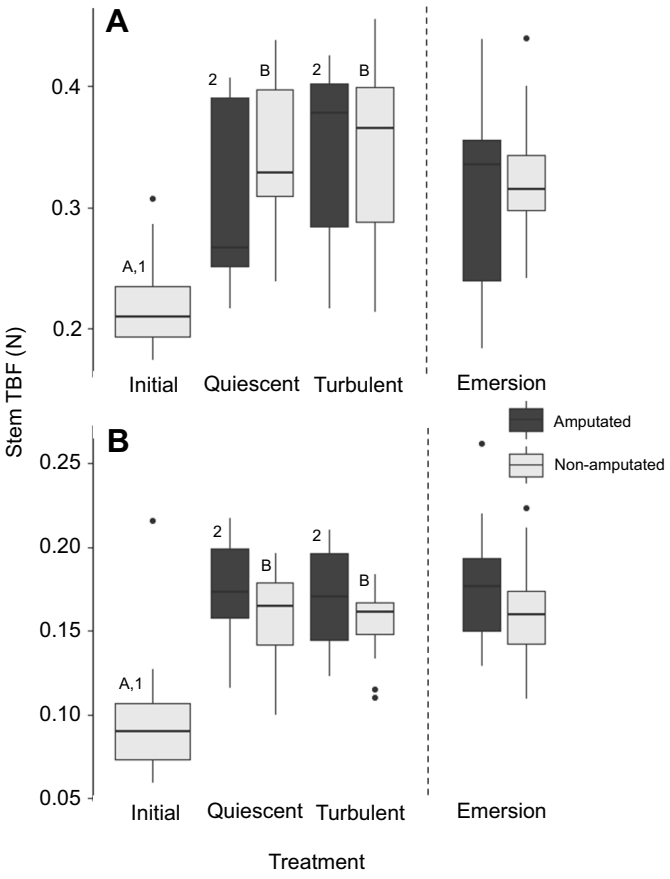

Fig. 6. Stem tensile breaking force (TBF) of initial and experimental sea urchins. Experimental sea urchins were subjected to hydrodynamic conditions (quiescent and turbulent) and amputation (amputated, non-amputated); those in the initial group did not undergo tube foot amputation. Stem TBF was evaluated in tube feet in the oral (A) and aboral (B) body locations. Statistical analyses excluded the emersion treatment, but measured values are included for reference. The boxplot horizontal line is the median, box edges are the 25th and 75th percentiles; whiskers indicate the largest value within 1.5 times the IQR and points beyond are values greater than 1.5 times the IQR but less than 3 times the IQR. Tukey *post hoc* test results between initial sea urchins and non-amputated tube feet from experimental sea urchins are presented with uppercase letters. Tukey *post hoc* test results between initial and amputated tube feet from experimental sea urchins are presented with numbers. Body locations had significantly different TBF (oral>aboral).

during the breeding season (Maginnis, 2006). Moreover, in purple sea urchins, spine regeneration has been associated with decreased gonad production (Haag et al., 2016). Studies addressing the physiological allocation of resources during tube foot regeneration are needed to better understand the potential ecological consequences of this important adaptive process.

Contrary to our prediction, the treatments used in this study did not stimulate regeneration of tube foot discs with areas comparable to those of non-amputated tube feet or pre-amputation tube feet. This could be due to the long regeneration time. In the field, amputated tube foot discs may similarly take a long time to regenerate to their original size and sea urchins rely on a surplus of tube feet to attach to the substrate, minimizing the effect of slow tube foot regeneration. Indeed, not all tube feet attach to the substrate at a given moment and sea urchins put down more tube feet when faced with increasing water velocity (Santos and Flammang, 2007). It is possible that plasticity in disc area under the different treatments results from differential growth rates and, given more time, amputated tube feet in all the treatments could have regrown to initial adhesive performance and morphology. However, a previous study failed to observe tube foot regeneration to initial morphology and functionality after 6 months in the laboratory (Narvaez et al., 2020). Alternatively, the regeneration of tube feet in the field may be significantly faster than observed in the laboratory. In the field, sea urchins may rely on multiple abiotic (i.e. hydrodynamics and emersion) and biotic (i.e. predation risk) cues. Without multiple cues in the laboratory, it is possible tube foot disc area regeneration was not maximized. Sea urchins in the field are also subjected to abiotic stressors of higher intensity than used in this study, which may be another reason regenerated tube foot discs in the laboratory were smaller than those of non-amputated or pre-amputated tube feet. The water velocity sea urchins experienced in the turbulent water treatment was between 0.03 and 2 m s^{-1}, but water velocity in the field can be an order of magnitude higher depending on habitat and depth (Denny, 1988). Thus, it is possible that in laboratory conditions, treatments of higher intensity or a combination of treatments could result in tube foot regeneration that matches pre-amputation disc area values. However, recreating ecologically relevant environmental stimuli, e.g. intense hydrodynamic forces, in the laboratory can be challenging, particularly when the stimuli must be maintained over a long period.

The treatments used in this study failed to stimulate differential tube foot maximum tenacity in regenerating an intact tube foot. However, a previous study conducted on *P. lividus* reported a decrease in the expression of the adhesive protein when sea urchins were kept in aquaria, suggesting its expression may be regulated by hydrodynamic conditions (Toubarro et al., 2016). Unavoidable delays in the shipment of the sea urchins to our facility kept them in laboratory conditions at Bodega Bay, CA, USA, for 2 weeks before our initial maximum disc tenacity was measured. Thus, maximum disc tenacity may have decreased in response to captivity, but we could not quantify it. We are unsure why the turbulent water movement treatment did not stimulate higher maximum disc tenacity, but as with disc area, it may be related to obtaining cues from multiple stressors or stressors with higher intensities. Alternatively, it could be that disc maximum tenacity in *S. purpuratus* is not affected by the environmental factors tested in this study. Studies assessing the plasticity of the expression of the adhesive protein in response to different abiotic and biotic factors, and on different sea urchin species are lacking.

Tube foot length was similarly unaffected by treatment: amputated tube feet failed to return to pre-amputation values and non-amputated tube feet maintained their initial length. Previous studies of sea urchin tube feet regenerating in laboratory conditions found similar results, where tube foot length did not recover to pre-amputation values (Bodnar and Coffman, 2016; Narvaez et al., 2020; Reinardy et al., 2015). These results further support the hypothesis that sea urchin tube foot regeneration is naturally slow, and successful adhesion to the substrate may be driven by the number of tube feet, not quality. In ophiuroids, arm regeneration is also slow, particularly in cold-water species (Clark et al., 2007). Thus, natural differences in sea urchin tube foot length in the field may be a result of slow regeneration after amputation and not the result of active plasticity. Alternatively, tube foot length may exhibit active plasticity related to different stressors, a combination of stressors, or to stressors of higher intensity than used in our laboratory experiment. In ophiuroids, for example, some species regenerated shorter and lighter arms when subjected to water motion in laboratory conditions (McAlister and Stancyk, 2003), while

others regenerated longer and heavier arms on seagrass beds exposed to higher wave action (Clements et al., 1994).

Stem TBF was not influenced by treatment or amputation but was higher at the end of the experiment than at the beginning. We hypothesize that the increase in sea urchin tube foot TBF throughout the experiment is due to a passive plasticity response to the movement from the field to the laboratory. Specifically, it is possible that our initial measure of TBF was negatively influenced by the delay in shipping and/or handling and shipping of sea urchins at the beginning of the experiment but recovered by the end of the experiment. Alternatively, these results could be explained by the access to high-quality food associated with laboratory conditions when compared with the field. In this experiment, sea urchins were fed *ad libitum* with kelp. However, in the habitat where they were collected (barren grounds), access to kelp is restricted and sea urchins are usually in poor nutritional condition (Lang and Mann, 1976). Sea urchins respond to starvation by reducing gut tissue and gonad mass (Guillou et al., 2000; Lares and Pomory, 1998). A similar phenomenon may occur with tube feet, with a lower mass in the tube feet resulting in a lower breaking force. To better understand the response of the stem in regenerating tube feet, future studies should measure material properties of the stem, such as modulus, by better controlling strain rate and applied stress, and providing measured stem area.

Our results are consistent with previous laboratory studies that failed to observe tube foot length and disc area regeneration to pre-amputation values (Bodnar and Coffman, 2016; Narvaez et al., 2020; Reinardy et al., 2015). We also found that the disc area of non-amputated tube feet showed a non-significant tendency to decrease in the quiescent treatment. This is consistent with a previous study that found the disc area of sea urchin tube feet decreased significantly after 6 months in the laboratory (Narvaez et al., 2020). The short duration of our study (4 months) likely explains the lack of a statistically significant difference; however, the change in area was in the expected direction. Together, these results suggest a plastic response of sea urchin tube foot disc area, length and TBF to standard laboratory conditions. These results bring up a relevant, but understudied, aspect of laboratory studies in invertebrates: the consequences of captivity on morphology, behavior and physiology.

The only study assessing the effect of novel settings on sea urchin behavior and physiological stress showed a negative impact of handling on both physiology and behavior (Bose et al., 2019). Our results suggest that laboratory studies using sea urchins should consider the effect of quiescent conditions, which are standard in laboratory settings, on phenotypic plasticity at the individual level and regeneration rates of their body parts. This consideration extends to other marine and aquatic invertebrates used as model organisms for laboratory regeneration studies, such as *Hydra* (Vogg et al., 2019) and sea stars (Carnevali, 2006); and to any invertebrate displaying high levels of phenotypic plasticity. Moreover, tube feet could have plastic responses to the characteristics of the artificial laboratory substrate to which they adhere, which in this experiment was PVC and plastic mesh. Indeed, a previous study found that *S. purpuratus* inhabiting pits created in mudstone and sandstone substrates have different disc sizes (Narvaez et al., 2020). In ecological studies, the discrepancy between laboratory, mesocosm and field studies has been a subject of interest for decades, particularly among scientists studying changes in community composition (Stachowicz et al., 2008) and the effect of climate change on communities, populations and organisms (Boyd et al., 2018; Stewart et al., 2013).

Tube foot amputation rates in the field are unknown for sea urchins, but previous studies have recorded hundreds of amputated tube feet when sea urchins are pulled from natural substrates with a harness (Stark et al., 2020). In ophiuroids, some species show signs of regeneration in up to 95% of their arms, and regeneration is strongly influenced by environmental conditions (Clark et al., 2007; McAlister and Stancyk, 2003). Future studies should focus on determining tube foot amputation rates in sea urchins collected in the field by assessing the scaring on tube foot stems (Lindsay, 2010) or by classifying tube feet within a certain percentile of length and disc size while regenerating. Assessing the incidence of tube foot loss in the field would shed light on the importance of the plastic response shown during the regeneration process.

Our results demonstrate a high degree of tube foot plasticity in response to different hydrodynamic conditions recreated in the laboratory. Observations of tube foot regeneration and plasticity in the field would reveal insights into whether any environmental cues result in the full restoration of tube foot morphology, which was not shown in the laboratory, and an active plastic response in tube foot performance. Anthropogenic carbon emissions increase the number, frequency and intensity of environmental stressors that marine organisms experience (Harley et al., 2006). Thus, understanding the role of active and passive plasticity in providing organisms with physiological, morphological and behavioral advantages will strengthen our predictions about the consequences of these changes for organismal fitness and survival.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.A.N., A.J.M., D.F.S., A.Y.S., M.P.R.; Methodology: C.A.N., A.J.M., D.F.S., J.P.C., A.Y.S., M.P.R.; Formal analysis: C.A.N.; Investigation: C.A.N., A.J.M.; Resources: C.A.N., D.F.S., A.Y.S., M.P.R.; Data curation: C.A.N., A.J.M.; Writing - original draft: C.A.N., A.J.M.; Writing - review & editing: C.A.N., A.J.M., D.F.S., J.P.C., A.Y.S., M.P.R.; Visualization: C.A.N.; Supervision: C.A.N., M.P.R.; Project administration: C.A.N., A.J.M.; Funding acquisition: M.P.R.

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