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Abstract

Animals clinging to natural surfaces have to generate attachment across a range of surface roughnesses in both dry and wet conditions. Plethodontid salamanders can be aquatic, semi-aquatic, terrestrial, arboreal, troglodytic, saxicolous, and fossorial and therefore may need to climb on and over rocks, tree trunks, plant leaves and stems, as well as move through soil and water. Sixteen species of salamanders were tested to determine the effects of substrate roughness and wetness on maximum cling angle. Substrate roughness had a significant effect on maximum cling angle, an effect that varied among species. Substrates of intermediate roughness (asperity size 100 µm to 350 µm) resulted in the poorest attachment performance for all species. Small species performed best on smooth substrates, while large species showed significant improvement on the roughest substrates (asperity size 1000 µm to 4000 µm), possibly switching from mucus adhesion on a smooth substrate to an interlocking attachment on rough substrates. Water, in the form of a misted substrate coating and a flowing stream, decreased cling performance in salamanders on smooth substrates. However, small salamanders significantly increased maximum cling angle on wetted substrates of intermediate roughness, compared with the dry condition. Study of cling performance and its relationship to surface properties may cast light onto how this group of salamanders has radiated into the most speciose family of salamanders that occupies diverse habitats across an enormous geographical range.

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Introduction

Animals must generate and maintain attachment to biological and abiotic substrates for locomotion, feeding, and reproduction. This attachment can have ecological consequences when falling results in the animal becoming injured, being at additional risk of predation, or exerting time and energy to return to suitable habitat. As a result, animals have evolved versatile and diverse mechanisms of attachment to natural surfaces. Clinging to and climbing on inclined, vertical, and inverted substrates which may be rough or smooth can present challenges based on the size of the organism and the mechanism of attachment. Animals may possess numerous attachment structures of different sizes and attachment mechanisms which enable them to cling to and climb on both smooth and rough surfaces over a large range of asperity sizes under different moisture conditions (Hanna and Barnes 1991; Zani 2000, 2001; Drechsler and Federle 2006; Federle et al. 2006; Huber et al. 2007; Persson 2007a; Niewiarowski et al. 2008; Riskin and Racey 2010; Riskin and Fenton 2011; Stark et al. 2012, 2013, 2015; Wainwright et al. 2013; Endlein, Barnes, et al. 2013; Endlein, Ji, et al. 2013; Crandell et al. 2014; Ditsche and Summers 2014; Ditsche et al. 2014; Drotlef et al. 2014; Beckert et al. 2015; Crawford et al. 2016; Wang et al. 2016; Stark and Yanoviak 2018; Langowski et al. 2019; Pillai et al. 2020).

Plethodontid salamanders access elevated and vertical habitats (McEntire 2016) and have been documented climbing on tree trunks, cave walls, and rock faces, in addition to surmounting obstacles such as boulders, tree trunks, and steep slopes while traversing forest floors, streambeds, and mountainsides (Wake 1987; Spickler et al. 2006; Camp et al. 2013). Climbing provides access to elevated or sheltered habitats where the temperature or humidity may be more suitable for adults or their offspring (Waldron and Humphries 2005; Spickler et al. 2006; Lunghi et al. 2014, 2017). Climbing also allows some species to move out of the reach of ground-dwelling predators or competitors (Huheey and Brandon 1973; Bury 2006; Crawford and Peterman 2013). Nighttime foraging up tree trunks and plant stems provides access to additional sources of prey (Jaeger 1978; Legros 2013). Salamanders have been found to be capable of clinging to smooth, dry laboratory substrates fully inverted (O'Donnell and Deban 2020, *in press*), but surfaces in nature are rarely smooth or dry.

Salamanders climb on both rough and smooth substrates. *Aneides* species climb redwood trees and limestone or sandstone outcroppings, where inversions and eversions of the bark or rock face reach the size of meters, down to the width and depth of salamander toes and smaller (Forsman and Swingle 2007; Smith et al. 2017). Bolitoglossan salamanders also live in trees, but some are found almost exclusively on smooth leaf surfaces in bromeliads and bananas (Alberch 1981; Green and Alberch 1981; Jaekel and Wake 2007; Blankers et al. 2012; Leenders and Watkins-Colwell 2013). *Eurycea lucifuga* and some *Hydromantes* species occupy natural caves in their native range, but also invade man-made mine shafts, water tanks, and drainage tunnels where the walls have been smoothed and shaped (Gorman and Camp 2006; Lunghi et al. 2014, 2015, 2017; Salvidio et al. 2015; Bradley and Eason 2018). Desmognathine salamanders cling to rock faces smoothed by water and fouled by plant material (Huheey and Brandon 1973), and terrestrial plethodontids in all habitats encounter obstacles on the forest floor including leaf litter, fallen trees, and exposed rock faces. While salamanders have been shown to climb extensively in nature, the role that foot morphology plays is uncertain (Jaekel and Wake 2007; Adams and Nistri 2010; Salvidio et al. 2015; McEntire 2016). Salamander foot morphology does not evolve in concert with arboreality, and for most plethodontid species investigated, no specialized toe-pad or foot-surface structures have been found (Green and Alberch 1981; Baken and Adams 2019). Clinging on surfaces of different roughness could present challenges to the formation of a frictional or adhesive bond of sufficient strength between the skin, mucus, and substrate, but roughness can also provide opportunities for salamanders to augment their attachment by interlocking with surface projections using the feet, toes, and tail.

Surface wetness is also a common feature of salamander habitats, which may impact the efficacy of attachment mechanisms which rely on intimate contact between the animal and the substrate, or between a biological adhesive and the substrate. In addition, the wettability of natural substrates varies from hydrophilic to hydrophobic and the wettability of a substrate has been shown to impact attachment strength in other taxa (Smith 1991; Neinhuis and Barthlott 1997, 1998; Bohn and Federle 2004; Anderson and Deban 2012; Stark et al. 2013, 2015; Voigt and Gorb 2017; Stark and Yanoviak 2018). As lungless salamanders, plethodontids are limited in their range and activity period to habitat, season, weather, or time of day when moisture levels ensure sufficient diffusion of oxygen across a moist skin surface (Beachy 1993; Peterman and Semlitsch 2014; Riddell and Sears 2015; McEntire 2016). For example, species of the genus *Desmognathus* can be found during the day clinging at angles of 90º (vertical) and higher on rock faces with flowing water in the Appalachian Mountains (Huheey and Brandon 1973; Crawford and Peterman 2013). Clinging to and climbing up misted and wetted surfaces is likely to be vital to lungless salamanders traversing their natural habitat.

We see varying effects of moisture on attachment in other taxa of climbing reptiles and amphibians, depending on the specific mechanism used and the substrate properties (Persson 2007b; Stark et al. 2012, 2013, 2015; Endlein, Barnes, et al. 2013; Ditsche and Summers 2014; Langowski, Dodou, et al. 2018; Stark and Yanoviak 2018). For example, tree frogs detach more easily from smooth acrylic substrates when a film of water is flowing down the surface, whereas torrent and rock frogs of the genus *Staurois* attach to rocks in fast flowing streams (Emerson and Diehl 1980; Endlein, Barnes, et al. 2013; Drotlef et al. 2014). Specialization in *Staurois* toe pad structure channels moisture and enhances attachment in these conditions (Endlein, Barnes, et al. 2013; Drotlef et al. 2014). In newts, *Cynops orientalis* showed the greatest stationary attachment on substrates in dry and lightly misted conditions (Wang et al. 2016). In geckos, studies of the effect of temperature, relative humidity, and amount of surface water demonstrated the complex effects of moisture on the attachment system, where setae and whole animal performance were enhanced, unaffected, or negatively impacted depending on the exact conditions, and differed across species (Huber et al. 2005; Niewiarowski et al. 2008; Puthoff et al. 2010; Prowse et al. 2011; Stark et al. 2012, 2013, 2015). In these cases, as in salamanders, attachment mechanisms may be tuned to the roughness and wetness of their preferred habitat or represent a multi-faceted approach that operates under diverse conditions.

Here we examine the effects of surface roughness and wetness on the ability of plethodontid salamanders to cling at a range of substrate inclinations (i.e., cling angles). We investigate how an increase in surface roughness affects species' maximum cling angle. In addition, we test how misted and flowing water affect attachment on smooth and roughened surfaces. We predict that roughened surfaces will allow species to engage in gripping behavior that will expand the range of angles on which clinging is possible. We also predict that species may show enhanced performance in wetter conditions that better match their microhabitat, due to tuning of the biological adhesive for high performance in their natural environment, but that flowing water could decrease cling performance by creating drag or disrupting the salamander's mucus coating. Studies of the effect of roughness and wetness on attachment performance across the Plethodontidae may help us to gain insight into links between morphology, habitat, and performance.

Methods

Animals

Animals were collected from populations in Chiapas, Mexico, and California and North Carolina, USA. Salamanders were housed individually in plastic enclosures on a substrate of damp unbleached paper towels at 16ºC to 21°C on a 12h/12h light schedule. Ambient moisture levels were standardized by controlling the amount of water added to enclosure paper towels to maintain 84 ± 10 % humidity. Species were fed on vitamin-dusted crickets or fruit flies, depending on size. Eighty-three individuals from 16 species (14 plethodontid and 2 ambystomatid species) were used in the study (*Ambystoma gracile*, *Ambystoma maculatum*, *Aneides flavipunctatus*, *Aneides lugubris*, *Aneides vagrans*, *Batrachoseps attenuatus*, *Bolitoglossa franklini*, *Desmognathus aeneus*, *Desmognathus ocoee*, *Desmognathus quadramaculatus*, *Ensatina eschscholtzii*, *Eurycea guttolineata*, *Eurycea wilderae*, *Plethodon elongatus*, *Plethodon metcalfi*, and *Pseudotriton ruber* (Table 1). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Florida.

Roughness

Plates with roughness asperities ranging from 0 to 4000 μm were fabricated from epoxy resin by forming negative molds of silicone rubber (Platinum Silicone Rubber, Smooth-On Mold Star Series) on roughened substrates of selected granule sizes and then casting epoxy resin (Crystal Clear Bar Table Top Epoxy Resin, Pro Marine Supplies) into them. Manufacturer's recommended molding and casting directions were followed. For the smooth plate (S), epoxy resin was poured into an aluminum baking tray to cure. For plates of intermediate roughness (I1, 100 – 150 μm and I2, 300 – 350 μm), the silicone rubber molds were poured over sandpaper of grit size P120 and P60 (Red Resin Power Sandpaper, Gator Power). For plates of highest roughness (R1, 1000 μm – 2000 μm and R2, 2000 – 4000 μm), gravel was filtered through soil sieves to select gravel of the desired diameter, then the silicone rubber was poured over custom constructed sheets of this densely packed gravel glued to cardboard. In all cases, epoxy resin was cast into the silicone rubber molds set into aluminum baking trays to form clinging substrates.

Epoxy resin plates were mounted to a wooden frame, and hung between two tripods to allow them to rotate 180º during experiments.

Clinging Procedure

Animals were placed on the clean, dry roughness plate suspended between two tripods (MeFoto Roadtrip Aluminum Travel Tripod) at an angle of 0 degrees (horizontal) (Fig. S1). All animals were oriented in the same direction, resulting in a head-up orientation throughout rotation. The angle to the nearest degree at which the animal detached was measured using a digital inclinometer (Wixey WR300BT). Animals were replaced in enclosures between trials to limit desiccation and prevent altered adhesive performance due to drying. Substrates were cleaned with ethanol and allowed to air dry between all trials.

Animals were tested in no more than three trials per day with a rest of at least three hours between trials. Trials in which animals reoriented to head downward position or voluntarily jumped off were not recorded or analyzed. The order of treatments was randomized. Each individual was tested in five trials per roughness and three per wetness treatment. Salamanders were measured to determine body mass in grams, snout-vent length in millimeters, and total body length in millimeters (measured from tip of the jaw to tip of the tail (Table S1).

Wetness

Experiments were conducted in the same manner as roughness treatments, using the same epoxy resin roughness plates, with sixty-seven individuals from 14 species. Individuals from *P. elongatus* and *A. gracile* were not available for the wetness experiments. Data in the flowing water treatment condition could not be collected from *E. guttolineata* and *A. vagrans* because they were in use in other experiments. Only roughness treatments of 0 µm, 300 – 355 µm, and

2000 – 4000 µm (S, I1, R2) were used in wetness trials, here referred to as “smooth,” “intermediate,” and “rough”.

The three wetness treatments consisted of a dry control, a misted treatment, and a flowing water treatment. The misted treatment consisted of water aerosolized and dispersed across the entire surface resulting in an even coating of individual droplets 0.1 to 0.01 mm in diameter. The flowing water treatment was created using perforated tubing affixed to the upper edge of the roughness plate. The tubing was connected to a 20 L water reservoir and, when gravity fed, released water across the roughness plate (Endlein, Barnes, et al. 2013) at a rate of 19-21 ml/s and a depth of 1 – 2 mm on the surface (Fig. S1).

For the misted treatment, salamanders were placed on a pre-misted substrate, such that the droplets were between the salamander mucus layer and the substrate. For the flowing water treatment, initial attempts to place salamanders on a smooth horizontal substrate already flooded with flowing water elicited immediate lateral undulation swimming movements and no clinging behavior. Therefore, salamanders were placed onto a clean, dry, horizontal substrate and then the water flow was started. After flowing water contacted the body of the salamander, the substrate was rotated by hand at a rate of 3º per second until the animal detached or until fully inverted (angle of 180º). As a result of this variation in the procedures for the two wetness experiments (misted water applied before salamander, flowing water initiated after salamander placement), it is not suitable to compare these two treatments with each other. Instead, both are compared solely with the dry treatment.

Analyses

All statistical analyses were conducted in R 3.5.2 (R Core Team 2019) in R Studio (R Studio Team 2015). Maximum cling angle data were tested for normality and homogeneity of variance using Shapiro-Wilks test and Levene's test, respectively. Individuals' maximum cling angle data were non-normal and heteroscedastic due to the extremely high number of 180º cling trials from high performing species. Analyses of significant differences were conducted between select treatments (Roughness S to I2, I2 to R2, and S to R2, as well as dry condition to misted condition and dry condition to wetted condition for all three roughness pairings) within species using pairwise, two-sample, two-tailed Wilcoxon Signed Rank tests for non-parametric data and corrected for false discovery rate (Benjamini and Hochberg, 1995) (Table S4 and S5). Treatments were selected because cling angles on roughness levels I1 and R1 were not significantly different from I2 and R2, respectively. Due to the non-parametric testing used, and the non-linear response of cling performance to increasing roughness, phylogenetic comparative methods and a comparative analysis of cling performance across species were not conducted. Instead all analyses conducted compared performance within a species across treatment conditions. Data from *E. guttolineata* were excluded from analyses due to limited numbers of individuals (N = 2).

Results

Roughness

Individuals from all 16 species tested were able to cling to dry surfaces across the full range of roughness treatments (asperity size 0 – 4000 μm) at maximum angles ranging from 76 ± 2º (mean and s.e.m.) to 180 ± 0º degrees from the horizontal (Table S1). On the smooth and intermediate roughness plates (S, I1, I2) salamanders were unable to engage in gripping with the toes and apparently attached via their mucus layer. In some cases, large salamanders (*A. gracile, A. maculatum, D. quadramaculatus, E. eschscholtzii,* and *P. ruber*) failed to cling at angles less than 90º due to sliding, a failure due to high shearing forces overwhelming the friction that attaches them to the substrate. At higher roughness (R1 and R2) some salamanders were observed augmenting their mucus attachment with gripping behaviors with the feet, toes, or tail, inserting them into gaps between asperities or interlocking with projections.

The relationship between maximum cling angle and roughness was non-linear and the effect of roughness on maximum cling angle varied among species in magnitude but not direction (Fig. 1). Phylogenetic comparative analyses of performance across species were not conducted due to issues of normality, heteroscedascity, and non-linearity, but differences in maximum cling angle within species across treatments were analyzed, and when the findings for each species were considered, certain trends emerged. Small species (body mass 0.6 g to over 5 g) clung better to smooth surfaces than large species (5g to 29.0 g, Table S2, Fig. 1). Overall, the effect of increasing roughness of the substrate resulted in significantly decreased cling angle (Fig. 1, Table S2) in nine of the 16 species when moving from smooth substrates to intermediate substrates, with significantly improved maximum cling angle from intermediate to highly roughened substrates in 12 of 16 species.

Species differed in whether maximal cling angle occurred on the rough (R2) or on the smooth (S) substrate (Fig. 1, Table S2) and in eight of 16 species, maximum cling angle on smooth (S) and highly rough (R2) substrates did not significantly differ (Table S4). For nine of the 16 species, the highest cling angle was achieved on smooth substrates, but large-bodied or dexterous species (*A. gracile, A. maculatum, A. flavipunctatus, A. lugubris, A. vagrans,* and *E. eschsholtzii)* clung significantly better to the roughest substrates (Fig. 1, Table S2, S4).

Wetness

All 14 species tested under wetness conditions were able to cling to dry, misted, and wetted roughness plates (S, I2, R2) at angles from 47º ± 6º to 180º ± 0º (Table S3, Fig. 2 - 4). In misted and flowing water on smooth and intermediate substrates, salamanders appeared to engage in mucus attachment with their ventral surface pressed against the surface, but with their feet not always in contact with the surface (Fig. S1). On the roughest substrate, we observed interlocking the feet and toes with projections on the surface, and in some cases, the ventral trunk was completely off the surface (Fig. S1). Water negatively impacted maximum cling angle on smooth surfaces in eight of 14 species, although seven of 14 species were more impacted by flowing water, and only four of 14 species were significantly impacted by misted water (Fig. 2).

Water significantly improved cling angle in six of 14 species on the intermediate (I2) substrate (Fig. 3, Table S3), although the magnitude of the effect varied by species (Table S5). Cling angle on the roughest plate (R2) was not significantly affected by any wetness treatment for 12 of the 14 species, except *D. aeneus* which clung better to flowing-water-covered surfaces and *A. maculatum* which clung poorly to misted roughened surfaces (Fig. 4, Table S5).

Discussion

Effect of roughness

The smooth surface negates any possibility of interlocking with the toes and feet. Large species such as *A. maculatum, A. flavipunctatus, A. lugubris, D. quadramaculatus, E. eschscholtzii, P. metcalfi,* and *P. ruber* detach at angles between 90° and 161°, indicating the attachment force is insufficient to resist the increasing component force of gravity acting normal to the surface, which increases non-linearly with respect to angle. Large species are particularly limited in any attachment determined by contact area due to their lower surface area to volume ratio (Schmidt-Nielsen 1975) and so they experience large shear and normal forces yet have the smallest attachment surface per unit body mass. All the species that experienced failure at angles below 90º, as a result of shearing detachment forces exceeding frictional attachment forces, were large in size (5.1 ± 0.8 g to 29.0 ± 3.0). Species that can cling at or near 180º (*D. aeneus, E. wilderae, B. attenuatus, D. ocoee, E. guttolineata, A. vagrans, P. elongatus,* and *B. franklini*), appear to have sufficient adhesive attachment between their ventral mucus layer and the surface to support their full body weight, even on a smooth substrate.

Poor attachment to intermediate substrates has consistently emerged in studies of cling performance in animals, but the scale on which this occurs is highly dependent on the size of the animal and their attachment mechanism (Emerson and Diehl 1980; Huber et al. 2007; Voigt et al. 2008; Scholz et al. 2010; Bullock and Federle 2011; Hosoda and Gorb 2011; Wolff and Gorb 2011; Langowski, Schipper, et al. 2018; Langowski et al. 2019; Pillai et al. 2020). Many vertebrate and invertebrate species for which clinging and climbing play major roles in their natural history use two or more attachment mechanisms (Zani 2000; Bullock and Federle 2011; Wolff and Gorb 2011; Nadler et al. 2013; Beckert et al. 2015; Labonte et al. 2016). Having two attachment mechanisms may increase the range of surfaces on which animals are able to maintain attachment, but surfaces that fall in between optimal conditions for both mechanisms can result in declines in performance.

The decline in salamander maximum cling angle on intermediate substrates may represent a critical roughness where one mechanism of attachment begins to fail but a secondary mechanism has not yet reached sufficient functionality, as has been shown in other organisms (Persson and Gorb 2003; Huber et al. 2007; Bullock and Federle 2011; Wolff and Gorb 2011). In salamanders, this transition point may occur because of a loss in mucus contact area, which can be highly effective on smooth substrates (O'Donnell and Deban 2020 *in press,* Fig. 1). One explanation is that the small irregularities in the intermediate substrate resulted in gaps in the attachment between the surface and the salamanders' ventral mucus coating. These small gaps between the mucus coating and the surface decreased the effective contact area of the animals. The reduction in maximum cling angle on intermediate substrates also suggests the mucus coating on the salamanders is either very thin, very viscous, or both, because it apparently did not flow into these gaps to increase contact area to the amount present on the smooth surface.

Interlocking of toes, feet, or tail can occur when the surface becomes rough enough (Cartmill, 1985). Large-bodied and dexterous species such as *A. maculatum, A. flavipunctatus, A. lugubris,* and *E. eschscholtzii* showed significant improvement on the roughest treatment (R2) over the intermediate (I2) and smooth (S) treatments (Table S2, S4, Fig. 1). In addition to being the largest species, these species also have some of the largest feet. The size of the grit elements relative to foot and toe size enable these species to engage in interlocking toes in crevices to enhance attachment (as in small species), but potentially also to use the entire foot to create attachment by antagonistic clasping of the toes around large granules (Cartmill, 1985).

Extremely small salamanders, such as *B. attenuatus, E. wilderae,* and *D. aeneus* showed an adhesive contact attachment on smooth (S) substrates where frequently their feet were not in contact with the surface during clinging. They were also able to engage in interlocking attachment on rough (R1 and R2) substrates to support their body weight (Fig. S1). Despite their smaller body size, feet, and toes, they did not show an ability to engage in interlocking at smaller levels of roughness (I1 and I2). Even with miniatured and reduced limbs, as in *B. attenuatus*, all tested species could fully support their weight on rough substrates when hanging from only the feet (Fig. S1). Broadly, both a reduction in cling angle on substrates of intermediate (I1) roughness and the ability to engage in interlocking attachment on substrates of sufficient roughness (R2) occurred in all species.

Effect of wetness

Water on the surface can either improve or reduce the cling performance of an animal, depending on the wettability of the substrate, mechanism of attachment, the depth of the water layer, and the force of the flow (Emerson and Diehl 1980; Stark et al. 2012, 2015; Endlein, Barnes, et al. 2013; Ditsche and Summers 2014; Wang et al. 2016; Stark and Yanoviak 2018). This effect has been found in frogs and newts, where in some species maximum cling angle on slightly roughened substrates is improved by the presence of water, increasing it to similar levels as on smooth dry substrates (Barnes et al. 2002; Endlein, Barnes, et al. 2013; Drotlef et al. 2014; Wang et al. 2016). However on smooth surfaces, fast-flowing water can pull the organism along the surface, rendering clinging impossible (Emerson and Diehl 1980). Epoxy resin is generally hydrophobic, as are some natural substrates (Neinhuis and Barthlott 1997), which can impact the attachment strength of the mucus, and the interaction between salamander mucus, water, and the surface energy of the substrate is not known at this time.

On the smooth plate, misted water negatively impacted species over 1g in mass, suggesting that having a dispersed layer of misted water between in the salamander and the surface acted to disrupt the frictional and adhesive properties of the mucus. For all species, maximum cling angle on the smooth substrate in flowing water was reduced to 96 ± 23°, suggesting moisture has a major impact on frictional attachment for many species. In the case of flowing water, the salamander was in contact with the surface before the water was introduced, suggesting that shearing forces were sufficient to overcome the skin-mucus-substrate bond and cause detachment.

On intermediate substrates, the misted treatment showed either comparable or improved attachment for all species over the dry. This improvement was strongest in the small species, *B.*

attenuatus, E. wilderae, D. aeneus, D. ocoee, and the larger, high performing *B. franklini* (Table S3, S5, Fig. 3). The surface irregularities of the intermediate treatment may have caused reduced contact area in the dry condition, and in the misted condition those gaps may be filled by the water droplets, as has been suggested for the secretions in some insect smooth adhesive pads, and with mucus or water in some frogs (Barnes et al. 2002; Drechsler and Federle 2006; Endlein, Barnes, et al. 2013). When the salamander ventral mucus layer comes into contact with the slightly wetted surface, the water may serve to increase the contact area of attachment over the dry condition. However, there is some indication of reduced adhesion strength compared with a smooth dry surface; this mucus and water attachment only restored maximum cling angle to the level of peak performance in the smallest species.

Many species showed improved maximum cling angle in flowing water on the intermediately roughened substrate; in addition to the species improved by the misted condition, which remained high performing in flowing water, larger species *P. metcalfi* and *E. eschscholtzii* also showed improvement in the flowing water over the dry treatment. Flowing water may play a similar role to misted water in filling contact area gaps for small species. In the case of *Ensatina*, their elevated posture while standing and clinging with little ventral body surface in contact results in extremely low contact area (O'Donnell and Deban 2020, *in press*). The flowing water may have the effect of triggering these species to crouch, increasing their contact area, or of filling some portion of the gap between their body and the surface.

On the roughest plate (R2), dry, misted, and flowing water conditions, cling angle was unaffected for nearly all species. This indicates that for most species the combination of interlocking attachment and remaining contact area on rough substrates is strong; any negative impact on the mucus layer from misted water coating the surface or shear forces created by the flowing water condition are not sufficiently strong to overcome interlocking attachment. Nor did water introduce enough additional lubrication of the surface projections to significantly decrease performance, except in the case of *Ambystoma*, the largest, heaviest, and poorest clinging species. Unlike in the intermediate treatment, water was evidently not needed on the rough substrate to improve attachment over the dry condition. In fact, many individuals hung purely by their feet on the roughest treatment, using little adhesive contact area in their attachment.

Conclusions

Salamanders cling on roughened and wetted substrates apparently using a combination of mucus-driven wet adhesion and interlocking the toes with surface projections. Salamanders cling to 180º on both smooth (S) and rough (R1 and R2) substrates but experience a decline in maximum cling angle at a critical roughness (I1 and I2). The decline at this critical roughness is likely caused by the decrease in adhesive contact area due to the asperity size of the intermediate substrates. It also suggests salamander mucus cannot flow into large grooves in roughened substrates to increase adhesive area. At greater roughness, declines in adhesive contact area can be compensated for with interlocking attachment, once asperity size is large enough to interlock with toes, and cling performance may even increase. For small salamanders under 4g, maximum cling angle (180º) occurs on both smooth (S) and rough substrates (R1 and R2). Large salamanders with poor maximum cling angle (90 – 165º) on smooth substrates have higher cling angle on rough substrates, where interlocking with the toes is possible, and these salamanders are capable of clinging fully inverted.

Water negatively affected salamander cling angle in some species across a range of surface roughnesses, but its effect was beneficial in some conditions. Misted and flowing water negatively affects cling angle on the smooth substrate compared with the dry condition but improves cling angle at the critical roughness (I2) for small species. Maximum cling angle of small salamanders on intermediate substrates (I2) with misted water matches performance on smooth, dry substrates, likely due to water filling in and bridging gaps between the body surface and the surface. For large species, misted water negatively affects performance on smooth substrates, and species are more likely to fail by sliding at angles between 0º and 90º. Flowing water, despite exerting larger drag forces than the misted condition, has a similar negative effect on smooth substrates to the misted condition, and has no effect on rough substrates, where interlocking is the major mechanism of attachment.

The robust performance of salamanders on many rough and wetted surfaces suggests that, in a range of moisture conditions, attachment on natural surfaces will be unaffected. Further research into the effect of substrate on clinging and climbing performance is needed to fully understand the mechanisms of attachment used by salamanders and the effect of naturally occurring degrees of roughness, wetness, and fouling. These data represent an effort to broadly test maximum cling angle in a large, diverse group of salamanders across several roughness treatments and wetness conditions. Future studies could quantify performance in a range of wetness conditions, including tests on materials with a range of surface energies and roughnesses that can be directly compared with natural surfaces.

Figures

Fig. 1 Effect of roughness on maximum cling angle. Cling angle in degrees for 16 species (Table S2) (in order of increasing body size from left to right, top to bottom) on substrates of increasing roughness (0 µm, 100 –150 µm, 300 – 355 µm, 1000 – 2000 µm, and 2000 – 4000 µm). Error bars indicate s.e.m., brackets denote pairwise comparisons between smooth (S) and intermediate (I2), between intermediate (I2) and rough, (R2) and between smooth (S) and rough (R2) substrates, asterisks denote significant differences ($P < 0.05$, Table S4).

Fig. 2 Effect of wetness on maximum cling angle on smooth substrates (S). Cling angle in degrees for 14 species (Table S3) on smooth substrates (S, 0 µm) under three wetness conditions (dry (D), misted (M), flowing water (W)). Error bars indicate s.e.m., brackets denote pairwise comparisons between dry and misted, and between dry and wetted conditions, asterisks denote significant differences ($P < 0.05$, Table S5).

Fig. 3 Effect of wetness on maximum cling angle on intermediately rough substrates (I2). Cling angle in degrees for 14 species (Table S3) on intermediate substrates (I2, 300 – 355 µm) under three wetness conditions (dry (D), misted (M), flowing water (W)). Error bars indicate s.e.m., brackets denote pairwise comparisons between dry and misted, and between dry and wetted conditions, asterisks denote significant differences ($P < 0.05$, Table S5).

Fig. 4 Effect of wetness on maximum cling angle on rough substrates (R2). Cling angle in degrees for 14 species of salamander (Table S3) on rough substrates (R2, 2000 – 4000 µm) under three wetness conditions (dry (D), misted (M), flowing water (W)). Error bars indicate s.e.m., brackets denote pairwise comparisons between dry and misted, and between dry and wetted conditions, asterisks denote significant differences ($P < 0.05$, Table S5).

Fig. S1 Images of salamanders clinging on rough and wet epoxy resin substrates, A) *Eurycea guttolineata* clinging at 180º to smooth (S) dry substrate. B) *Desmognathus aeneus* clinging to smooth (S) substrate at 180º with a misted coating of water, not using hindlimbs to attach. C) *Batrachoseps attenuatus* smooth (S) substrate at 180º with a misted coating of water. D) *Eurycea wilderae* clinging to smooth (S) substrate at 180º with a misted coating of water, not using forelimbs to attach. E) *Batrachoseps attenuatus* clinging to rough (R2) dry substrate at 180º using only the forelimbs and dangling the body and lengthy tail. F) *Eurycea wilderae* clinging to the intermediate (I2) substrate at 180º under flowing water conditions. G) *Desmognathus quadramaculatus* clinging to the intermediate (I2) substrate at 115º under flowing water conditions. H) *Aneides vagrans* clinging to the rough (R2) dry substrate at 180º using interlocking of the toes and tail.

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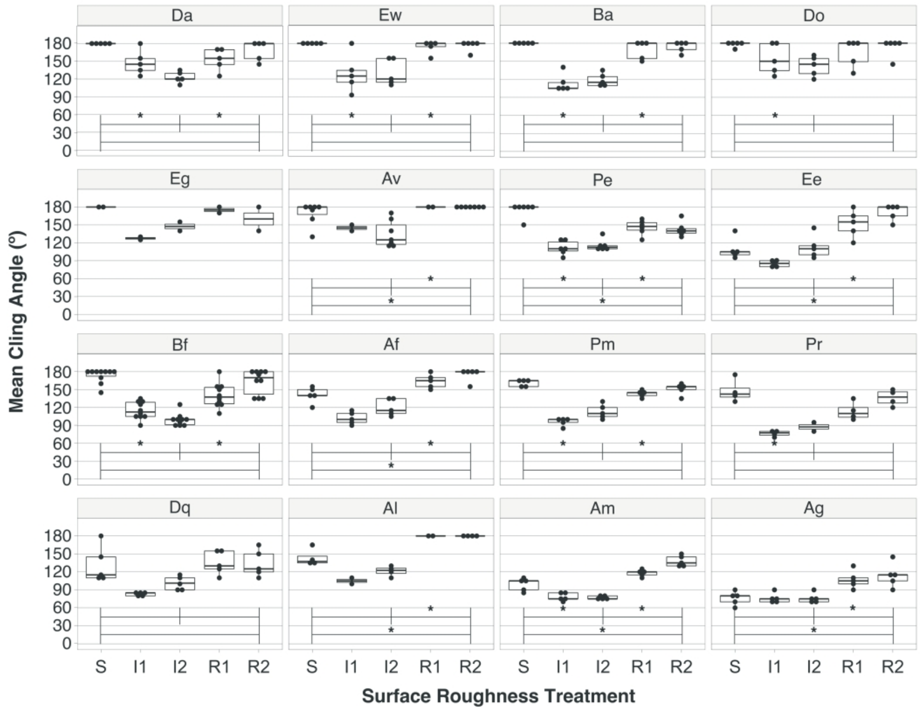

Fig. 1 Effect of roughness on maximum cling angle. Cling angle in degrees for 16 species (Table S2) (in order of increasing body size from left to right, top to bottom) on substrates of increasing roughness (0 μm, 100 –150 μm, 300 – 355 μm, 1000 – 2000 μm, and 2000 – 4000 μm). Error bars indicate s.e.m., brackets denote pairwise comparisons between smooth (S) and intermediate (I2), between intermediate (I2) and rough, (R2) and between smooth (S) and rough (R2) substrates, asterisks denote significant differences ($P < 0.05$, Table S4).

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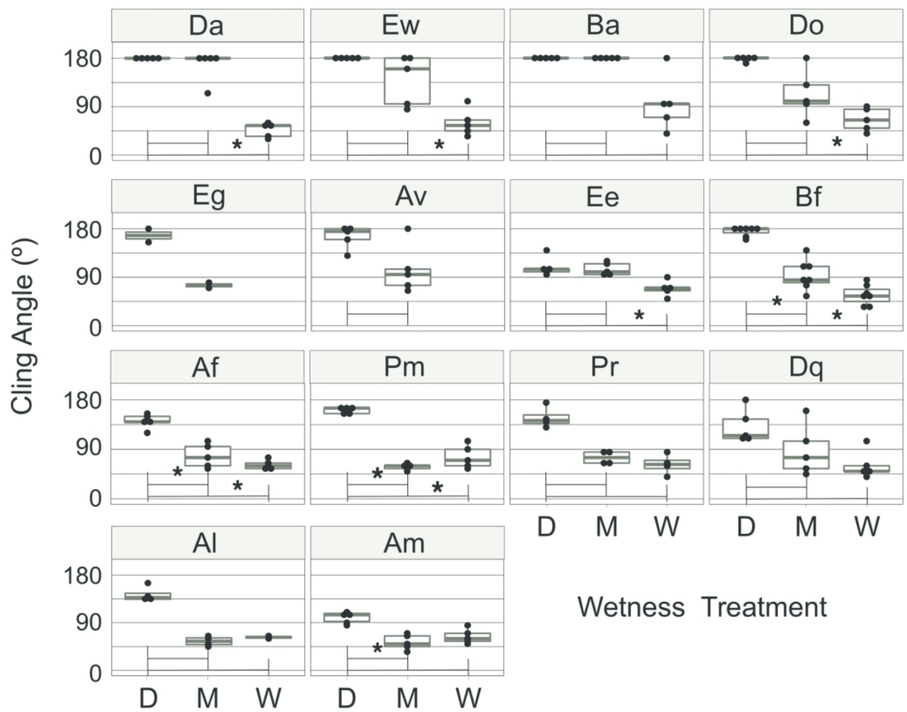

Fig. 2 Effect of wetness on maximum cling angle on smooth substrates (S). Cling angle in degrees for 14 species (Table S3) on smooth substrates (S, 0 μm) under three wetness conditions (dry (D), misted (M), flowing water (W)). Error bars indicate s.e.m., brackets denote pairwise comparisons between dry and misted, and between dry and wetted conditions, asterisks denote significant differences (P < 0.05, Table S5).

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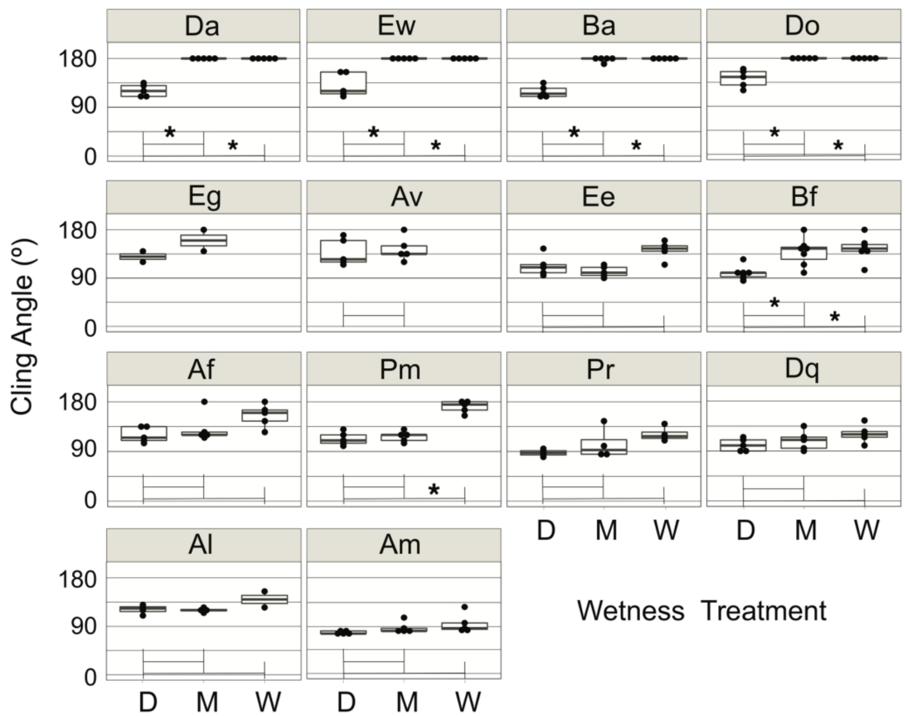

Fig. 3 Effect of wetness on maximum cling angle on intermediately rough substrates (I2). Cling angle in degrees for 14 species (Table S3) on intermediate substrates (I2, 300 – 355 μm) under three wetness conditions (dry (D), misted (M), flowing water (W)). Error bars indicate s.e.m., brackets denote pairwise comparisons between dry and misted, and between dry and wetted conditions, asterisks denote significant differences ($P < 0.05$, Table S5).

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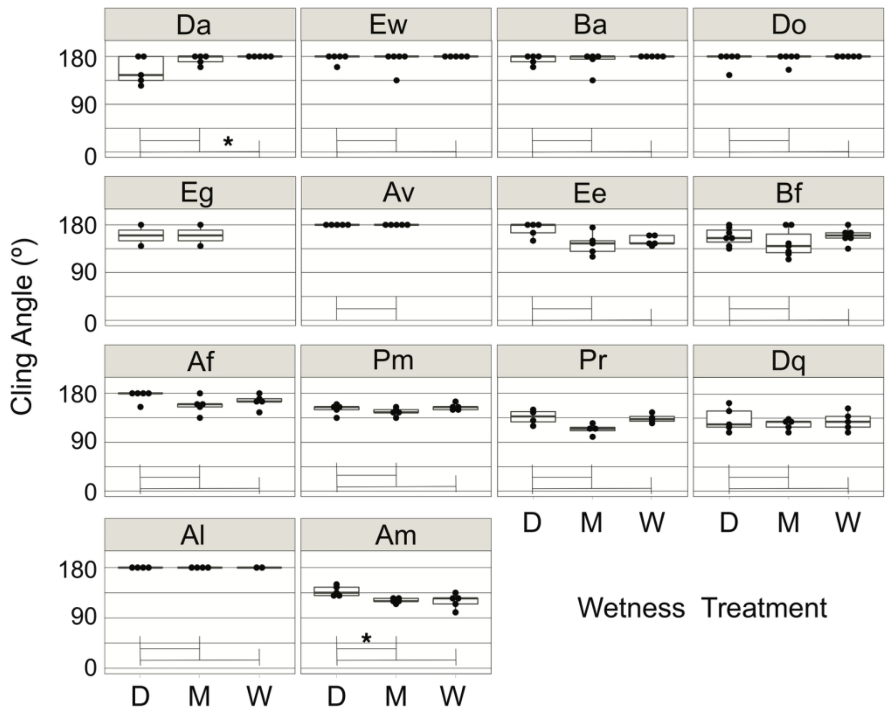

Fig. 4 Effect of wetness on maximum cling angle on rough substrates (R2). Cling angle in degrees for 14 species of salamander (Table S3) on rough substrates (R2, 2000 – 4000 μm) under three wetness conditions (dry (D), misted (M), flowing water (W)). Error bars indicate s.e.m., brackets denote pairwise comparisons between dry and misted, and between dry and wetted conditions, asterisks denote significant differences ($P < 0.05$, Table S5).

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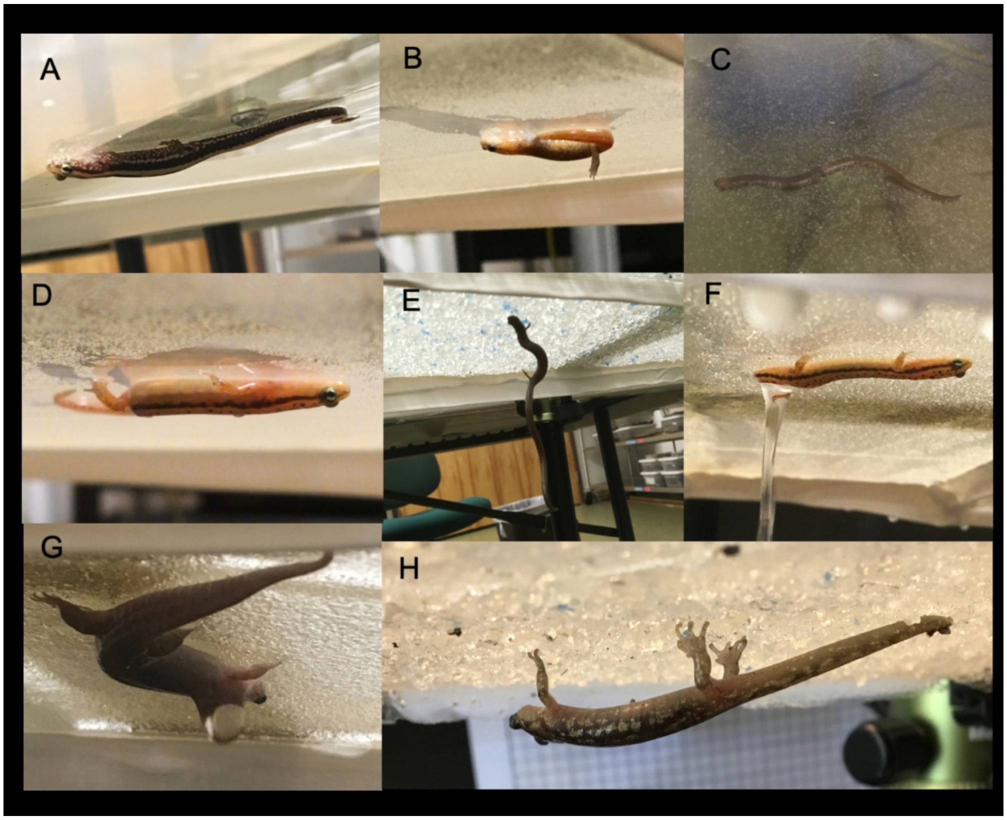

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Table 1 – Research Animals. Species' mean and s.e.m. for body mass, snout vent length (SVL), and tot from the tip of the jaw to the tip of the tail). Number of individuals tested in roughness experiments inc values indicate number of individuals tested in wetness experiments.

Species	Species Code	*N*	Body Mass (g)	SVL (mm)
Ambystoma gracile	Ag	5 (0)	26.4 ± 5.6	81 ± 8
Ambystoma maculatum	Am	5	29.0 ± 3.0	101 ± 5
Aneides flavipunctatus	Af	5	5.5 ± 1.9	59 ± 4
Aneides lugubris	Al	4	11.4 ± 2.0	87 ± 2
Aneides vagrans	Av	7 (5)	3.5 ± 0.6	46 ± 4
Batrachoseps attenuatus	Ba	5	0.8 ± 0.1	43 ± 2
Bolitoglossa franklini	Bf	10 (7)	7.0 ± 1.0	63 ± 3
Desmognathus aeneus	Da	5	0.6 ± 0.0	29 ± 1
Desmognathus ocoee	Do	5	2.2 ± 0.1	44 ± 1
Desmognathus quadramaculatus	Dq	5	16.4 ± 1.7	92 ± 3
Ensatina eschscholtzii	Ee	5	5.1 ± 0.8	58 ± 2
Eurycea guttolineata	Eg	2	0.9 ± 0.0	38 ± 0.1
Eurycea wilderae	Ew	5	0.8 ± 0.1	40 ± 2
Plethodon elongatus	Pe	6 (0)	2.3 ± 0.3	94 ± 4
Plethodon metcalfi	Pm	5	6.6 ± 0.3	70 ± 2
Pseudotriton ruber	Pr	4	12.1 ± 0.9	80 ± 2

tal body length (measured
dicated as *N;* bracketed

Total Body Length (mm)
176 ± 18
198 ± 8
110 ± 9
156 ± 3
87 ± 9
99 ± 9
109 ± 4
52 ± 2
89 ± 3
168 ± 6
112 ± 7
90 ± 1
91 ± 4
50 ± 3
146 ± 2
133 ± 5

Table 2 – Cling performance on roughened surfaces. Species' mean and standard error of the mean for m calculated from individual's maximum cling performance. Cling performance reported for each of three r Roughness treatment asperity or granule size was 0µm, 100 – 150 um, 300 – 355 µm, 1000 – 2000 um, a indicated. Number of individuals tested indicated as *N*.

			Roughness Treatm		
			0 µm	100 – 150 µm	300 – 355 µm
Species	Code	*N*	Cling Angle (°)		
Ambystoma gracile	Ag	5	76 ± 5	76 ± 4	76 ± 4
Ambystoma maculatum	Am	5	99 ± 5	78 ± 3	77 ± 1
Aneides flavipunctatus	Af	5	141 ± 6	102 ± 5	120 ± 6
Aneides lugubris	Al	4	144± 7	105 ± 5	121 ± 4
Aneides vagrans	Av	7	169 ± 7	145 ± 5	135 ± 8
Batrachoseps attenuatus	Ba	5	180 ± 0	114 ± 7	119 ± 5
Bolitoglossa franklini	Bf	9	174 ± 4	115 ± 5	100 ± 3
Desmognathus aeneus	Da	5	180 ± 0	148 ± 9	123 ± 4
Desmognathus ocoee	Do	5	178 ± 2	154 ± 11	142 ± 8
Desmognathus quadramaculatus	Dq	6	132 ± 14	83 ± 1	101 ± 5
Ensatina eschscholtzii	Ee	5	109 ± 8	85 ± 2	113 ± 9
Eurycea guttolineata	Eg	2	180 ± 0	128 ± 3	148 ± 8
Eurycea wilderae	Ew	5	180 ± 0	130 ± 14	131 ± 10
Plethodon elongatus	Pe	6	175 ± 5	112 ± 5	116 ± 4
Plethodon metcalfi	Pm	5	161 ± 2	96 ± 3	113 ± 5
Pseudotriton ruber	Pr	4	148 ± 10	76 ± 2	88 ± 3

ıaximum cling performance,
ɾoughness treatments.
ınd 2000 – 4000 μm, as

ıent	
1000 – 2000 μm	2000 – 4000 μm
)	
107 ± 7	114 ± 9
118 ± 3	138 ± 4
164 ± 5	175 ± 5
180 ± 0	180 ± 0
180 ± 0	180 ± 0
169 ± 7	174 ± 4
141 ± 6	163 ± 6
153 ± 8	168 ± 8
164 ± 10	173 ± 7
135 ± 9	134 ± 10
152 ± 10	171 ± 6
175 ± 5	160 ± 20
174 ± 5	176 ± 4
146 ± 5	143 ± 5
143 ± 4	151 ± 4
114 ± 8	136 ± 7

Table 3 - Cling angle on roughened, wetted surfaces. Species' mean and s.e.m. for maximum cling performance, calculated from individual's maximum cling performance. Cling performance reported for each of three roughness treatments, under three wetness regimes. Roughness treatment as indicated. Wetness treatment conditions as described in methods. Number of individuals tested indicated as *N*.

				Roughness Treatment		
				0 µm	300 – 355 µm	2000 – 4000 µm
Species	Species Code	*N*	Wetness Treatment	Cling Angle (°)		
Ambystoma maculatum	Am	5	Dry	99 ± 5	77 ± 1	138 ± 4
			Misted	53 ± 6	86 ± 5	121 ± 2
			Wet	64 ± 6	94 ± 8	120 ± 6
Aneides flavipunctatus	Af	5	Dry	141 ± 6	141 ± 6	141 ± 6
			Misted	78 ± 10	78 ± 10	78 ± 10
			Wet	62 ± 4	62 ± 4	62 ± 4
Aneides lugubris	Al	2	Dry	150 ± 15	115 ± 5	180 ± 0
			Misted	63 ± 3	118 ± 3	180 ± 0
			Wet	63 ± 3	140 ± 15	180 ± 0
Aneides vagrans	Av	5	Dry	165 ± 9	165 ± 9	165 ± 9
			Misted	104 ± 20	104 ± 20	104 ± 20
			Wet	-	-	-
Batrachoseps attenuatus	Ba	5	Dry	180 ± 0	119 ± 5	174 ± 4
			Misted	180 ± 0	178 ± 2	170 ± 9
			Wet	96 ± 23	180 ± 0	180 ± 0
Bolitoglossa franklini	Bf	7	Dry	173 ± 5	98 ± 4	162 ± 7
			Misted	94 ± 11	139 ± 10	145 ± 10
			Wet	57 ± 7	145 ± 8	159 ± 5
Desmognathus aeneus	Da	5	Dry	180 ± 0	123 ± 4	168 ± 8
			Misted	167 ± 13	180 ± 0	174 ± 4
			Wet	47 ± 6	180 ±0	180 ± 0
Desmognathus ocoee	Do	5	Dry	178 ± 2	127 ± 8	173 ± 7
			Misted	113 ± 20	180 ± 0	175 ± 5
			Wet	66 ± 10	180 ± 0	180 ± 0
Desmognathus quadramaculatus	Dq	5	Dry	128 ± 12	98 ± 5	131 ± 9
			Misted	88 ± 21	109 ± 8	125 ± 4
			Wet	61 ± 11	121 ± 7	131 ± 8
Ensatina eschscholtzii	Ee	5	Dry	109 ± 8	113 ± 9	171 ± 6
			Misted	105 ± 5	102 ± 5	144 ± 9
			Wet	69 ± 6	142 ± 8	150 ± 4
Eurycea guttolineata	Eg	5	Dry	168 ± 13	130 ± 10	160 ± 20
			Misted	75 ± 5	160 ± 20	160 ± 20
			Wet	-	-	-
			Dry	180 ± 0	131 ± 10	176 ± 4

Eurycea wilderae	Ew	5	Misted	140 ± 21	180 ± 0	171 ± 9
			Wet	60 ± 11	180 ± 0	180 ± 0
Plethodon metcalfi	Pm	5	Dry	161 ± 2	113 ± 5	151 ± 4
			Misted	58 ± 3	117 ± 4	146 ± 3
			Wet	76 ± 9	171 ± 5	155 ± 3
Pseudotriton ruber	Pr	4	Dry	148 ± 10	88 ± 3	136 ± 7
			Misted	75 ± 6	104 ± 14	114 ± 5
			Wet	63 ± 9	121 ± 7	134 ± 4

Table S4 - Statistical results of roughness tests. Pairwise, two-sample, two-tailed Wilcoxon Signed Rank tests for non-parametric data and corrected for false discovery rate (Benjamini and Hochberg, 1995). Test statistic D reported, as well as adjusted significance thresholds. Adjusted P values below 0.05 indicate significant differences in samples from the two compared treatments, either smooth (0 µm), intermediate (300 – 355 µm), or rough (2000 – 4000 µm) for that species.

Species	Test		Adjusted P Value
Aneides flavipunctatus	Smooth vs Intermediate	0.8	0.079
	Intermediate vs Rough	1	0.012
	Smooth vs Rough	1	0.012
Aneides lugubris	Smooth vs Intermediate	1	0.229
	Intermediate vs Rough	1	0.043
	Smooth vs Rough	1	0.043
Aneides vagrans	Smooth vs Intermediate	0.7	0.053
	Intermediate vs Rough	1	0.002
	Smooth vs Rough	0.9	0.012
Ambystoma gracile	Smooth vs Intermediate	0.4	0.873
	Intermediate vs Rough	1	0.012
	Smooth vs Rough	1	0.012
Ambystoma maculatum	Smooth vs Intermediate	1	0.008
	Intermediate vs Rough	1	0.008
	Smooth vs Rough	1	0.008
Batrachoseps attenuatus	Smooth vs Intermediate	1	0.012
	Intermediate vs Rough	1	0.012
	Smooth vs Rough	0.4	0.873
Bolitoglossa franklini	Smooth vs Intermediate	1	0
	Intermediate vs Rough	1	0
	Smooth vs Rough	0.3	0.787
Desmognathus aeneus	Smooth vs Intermediate	1	0.012
	Intermediate vs Rough	1	0.012
	Smooth vs Rough	0.4	0.873
Desmognathus ocoee	Smooth vs Intermediate	1	0.024
	Intermediate vs Rough	0.8	0.119
	Smooth vs Rough	0.2	0.357
Desmognathus quadramaculatus	Smooth vs Intermediate	0.7	0.214
	Intermediate vs Rough	0.8	0.078
	Smooth vs Rough	0.3	0.931
Ensatina eschscholtzii	Smooth vs Intermediate	0.4	0.873
	Intermediate vs Rough	1	0.012
	Smooth vs Rough	1	0.012
Eurycea wilderae	Smooth vs Intermediate	1	0.012
	Intermediate vs Rough	1	0.012
	Smooth vs Rough	0.4	0.357
	Smooth vs Intermediate	1	0.006

Plethodon elongatus	Intermediate vs Rough	0.8	0.026
	Smooth vs Rough	0.8	0.026
Plethodon metcalfi	Smooth vs Intermediate	1	0.012
	Intermediate vs Rough	1	0.012
	Smooth vs Rough	0.8	0.357
Pseudotriton ruber	Smooth vs Intermediate	1	0.024
	Intermediate vs Rough	0.8	0.119
	Smooth vs Rough	0.4	0.873

Table S5 - Statistical results of wetness tests. Pairwise, two-sample, two-tailed Wilcoxon Signed Rank tests for non-parametric data and corrected for false discovery rate (Benjamini and Hochberg, 1995). Adjusted P values below 0.05 indicate significant differences for that species between the two indicated wetness treatments (dry, misted, or flowing water) at that roughness treatment, either smooth (0 µm), intermediate (300 – 355 µm), or rough (2000 – 4000 µm) for that species.

Species	Roughness	Test	Test Statistic D	Adjusted P Value
Aneides flavipunctatus	Smooth	Dry vs Misted	1	0.023
		Dry vs Flowing Water	1	0.023
	Intermediate	Dry vs Misted	0.6	0.357
		Dry vs Flowing Water	0.8	0.159
	Rough	Dry vs Misted	0.6	0.357
		Dry vs Flowing Water	0.6	0.357
Aneides lugubris	Smooth	Dry vs Misted	1	0.086
		Dry vs Flowing Water	1	0.086
	Intermediate	Dry vs Misted	0.5	0.926
		Dry vs Flowing Water	0.8	0.343
	Rough	Dry vs Misted	0.3	1
		Dry vs Flowing Water	0.8	0.343
Aneides vagrans	Smooth	Dry vs Misted	0.8	0.238
		Dry vs Flowing Water	-	-
	Intermediate	Dry vs Misted	0.4	0.873
		Dry vs Flowing Water	-	-
	Rough	Dry vs Misted	0.4	0.873
		Dry vs Flowing Water	-	-
Ambystoma maculatum	Smooth	Dry vs Misted	1	0.024
		Dry vs Flowing Water	0.8	0.079
	Intermediate	Dry vs Misted	0.8	0.079
		Dry vs Flowing Water	0.8	0.079
	Rough	Dry vs Misted	1	0.024
		Dry vs Flowing Water	0.8	0.079
Batrachoseps attenuatus	Smooth	Dry vs Misted	0.4	0.873
		Dry vs Flowing Water	0.8	0.119
	Intermediate	Dry vs Misted	1	0.024
		Dry vs Flowing Water	1	0.024
	Rough	Dry vs Misted	0.4	0.873
		Dry vs Flowing Water	0.8	0.119
Bolitoglossa franklini	Smooth	Dry vs Misted	0.9	0.003
		Dry vs Flowing Water	1	0.001
	Intermediate	Dry vs Misted	0.75714	0.001
		Dry vs Flowing Water	0.85714	0.004
	Rough	Dry vs Misted	0.41429	0.454
		Dry vs Flowing Water	0.35714	0.56

Desmognathus aeneus	Smooth	Dry vs Misted	0.2	1
		Dry vs Flowing Water	1	0.012
	Intermediate	Dry vs Misted	1	0.012
		Dry vs Flowing Water	1	0.012
	Rough	Dry vs Misted	0.6	0.429
		Dry vs Flowing Water	1	0.012
Desmognathus ocoee	Smooth	Dry vs Misted	0.8	0.119
		Dry vs Flowing Water	1	0.016
	Intermediate	Dry vs Misted	1	0.016
		Dry vs Flowing Water	1	0.016
	Rough	Dry vs Misted	0.4	0.873
		Dry vs Flowing Water	0.6	0.429
Desmognathus quadramaculatus	Smooth	Dry vs Misted	0.6	0.536
		Dry vs Flowing Water	0.8	0.238
	Intermediate	Dry vs Misted	0.6	0.536
		Dry vs Flowing Water	0.8	0.238
	Rough	Dry vs Misted	0.2	1
		Dry vs Flowing Water	0.2	1
Ensatina eschscholtzii	Smooth	Dry vs Misted	0.2	1
		Dry vs Flowing Water	1	0.048
	Intermediate	Dry vs Misted	0.4	1
		Dry vs Flowing Water	0.8	0.159
	Rough	Dry vs Misted	0.6	0.536
		Dry vs Flowing Water	0.8	0.159
Eurycea wilderae	Smooth	Dry vs Misted	0.6	0.429
		Dry vs Flowing Water	1	0.016
	Intermediate	Dry vs Misted	1	0.016
		Dry vs Flowing Water	1	0.016
	Rough	Dry vs Misted	0.4	0.873
		Dry vs Flowing Water	0.8	0.119
Plethodon metcalfi	Smooth	Dry vs Misted	1	0.016
		Dry vs Flowing Water	1	0.016
	Intermediate	Dry vs Misted	0.4	0.873
		Dry vs Flowing Water	1	0.016
	Rough	Dry vs Misted	0.4	0.873
		Dry vs Flowing Water	0.4	0.873
Pseudotriton ruber	Smooth	Dry vs Misted	1	0.0571
		Dry vs Flowing Water	1	0.0571
	Intermediate	Dry vs Misted	0.5	0.9257
		Dry vs Flowing Water	1	0.0571
	Rough	Dry vs Misted	0.75	0.3429
		Dry vs Flowing Water	0.25	1