Pitviper thermography: Evoked behavioral assays indicate the pit organ used by crotaline snakes to detect thermal contrast has poor spatial resolution

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Summary Statement
Our experiments indicated thermal imaging by rattlesnake facial pits have poor resolution compared to thermographic cameras; consequently, patterned thermal backgrounds in natural environments may present a challenge for target detection.

ABSTRACT

Pitvipers detect infrared radiation by means of temperature contrasts created on their pit organ membranes. Signals from pit organs integrate with visual signals in the optic tectum, leading to the conjecture that the facial pits operate as an extension of the visual system. Because similar mechanisms underlie thermal imaging technology, imagery from thermal cameras is often used to infer how pitvipers perceive their environment. However, pit organs lack a focusing mechanism, and biophysical models predict that pit organs should have poor spatial resolution compared to thermal imaging cameras. Nevertheless, behavioral studies occasionally suggest pits may have better resolution than predicted by biophysical models, indicating that processing in the central nervous system may improve imaging. To estimate the spatial resolution of the neural image informing behavior, we recorded snake responses evoked by targets moving across backgrounds composed of two contrasting temperatures with an average temperature equal to the target temperature. An unresolved background would appear uniform; thus, the target would be detectable only if the background pattern were resolved. Western Rattlesnakes (*Crotalus oreganus*) displayed no statistically significant responses to targets presented in front of patterned backgrounds, regardless of the temperature contrasts or spatial frequencies within the background, but

responded strongly to targets presented in front of homogenous backgrounds. We found no evidence the pit organ system can resolve spatial details subtending an angle of 9 degrees or less. We discuss the implications of these results for understanding pit organ function in ecologically relevant habitats with thermal heterogeneity.

INTRODUCTION

The pitviper thermal imaging system has attracted considerable attention in both professional literature and popular media. Images and video recorded by commercial thermal imagers are often used to visualize what a pitviper might perceive (e.g., in den Bosch, 1983; Kardong, 1986; Rundus et al., 2007). However, there is reason to suspect that the high resolution of such images does not accurately simulate what pitvipers perceive. A better understanding of the quality of the neural image informing pitviper behavior is needed.

The loreal pit organ from which crotaline snakes derive their common name has long been known to be an integral aspect of their visual system (reviewed by Goris, 2011). Although the pit organ is structured as a lensless chamber eye similar to that of *Nautilus* (Land and Fernald, 1992), the optical anatomy and sensory physiology differ from those of visual systems (Goris, 2011). Most vertebrate and invertebrate eyes utilize a lens to focus electromagnetic radiation onto specialized pigments that absorb visible light (wavelength range ~ 400-800 nm). In contrast, the pit functions as a chamber eye with a pinhole aperture and a sensory membrane suspended in the pit. Rather than utilizing quantum detection, the membrane is heated by thermal infrared radiation (range ~ 5-20 μm). Radiation entering from different directions forms a temperature pattern sensed by receptors that respond to receptor temperature changes at least as small as 0.003°C (Bullock and Diecke, 1956). This sensitivity means that objects with temperatures contrasting by a few °C moving within its field of view and less than a few tens of meters distant create a detectable neural image of the changing temperature patterns on the membrane (Bakken, 2007; Bakken and Krochmal, 2007).

Neurologically, the sensory input from the temperature-sensitive membrane within the pit is processed through the lateral descending trigeminal tract (LTTD) and nucleus reticularis caloris (RC), integrated with sensory input from the lateral eyes in the optic tectum, and forwarded to the telencephalon (Berson and Hartline, 1988; Hartline et al., 1978). The hypothesis that a multispectral image is presented to the telencephalon is supported by this neurological anatomy and prey targeting disruption resulting

from contralateral blocking of eyes and pits (Chen et al., 2012). However, the spatial resolution of the resulting neural image that informs behavior is unknown.

The geometric optics of the loreal pit are defined by its morphology. The light energy from a source point that passes through the aperture falls on an area of the sensory membrane called the point spread function (PSF), measured in angular degrees. A small aperture creates a small PSF and thus a well resolved image, but admits little radiant energy. Enlarging the aperture admits more energy, but the larger PSF impairs resolution (Land and Fernald, 1992). The pit organ has a relatively large aperture which varies with view angle (Bakken et al., 2012; Kohl et al., 2012). Biophysical modeling indicates that, while detectable temperature patterns result, the angular resolution of the membrane temperature image is poor (Bakken and Krochmal, 2007). Bakken et al. (2012) found variation in pit morphology affecting membrane image resolution among 4 individuals of 4 species. The *Crotalus atrox* specimen had the highest resolution, *C.oreganus* and *C. horridus* intermediate, and the Asian white-lipped palm viper *Cryptelytrops albolabris* the lowest. However, even for *C. atrox*, the pit membrane resolution was extremely poor, and the sensory and ecological significance of the observed interspecific variation remains to be determined.

The neural image informing behavior is not necessarily defined by the pit membrane image. Computer image sharpening is a well-established technology, and it is theoretically possible that processing in the central nervous system could sharpen the membrane image (Sichert et al., 2006). Indeed, neurophysiological studies indicate that neural sharpening occurs in the LTTD of *C. oreganus,* and effectively reduces the effective aperture diameter (and thus the PSF) by half (Stanford and Hartline, 1980; Stanford and Hartline, 1984). See illustration in Fig. 1. Recent studies indicate that the LTTD and RC also process information from the pit organ to enhance contrast, sense motion, and determine directionality (Bothe et al., 2018; Bothe et al., 2019; Kaldenbach et al., 2016; Kohl et al., 2014). This merger might, for example, allow the optical input to train the neural network processing thermal information to further enhance resolution. However, a study of a congenitally one-eyed python suggested visual and thermal senses were ontogenetically independent in pythons (Grace and Woodward, 2001), and the predatory performance of a congenitally blind *C. oreganus* was nearly normal (Kardong and Mackessy, 1991). While these studies suggest pitvipers possess a multispectral sense combining visual and thermal input, the neurophysiological details of the entire system, including whether further thermal image sharpening occurs in the optic tectum and telencephalon, are not sufficiently known to estimate the acuity of the neural thermal images informing behavior.

Behaviors observed in free-ranging snakes have raised interesting questions related to the resolution of the neural thermal image. Snakes are most likely to detect and strike at prey that show stronger temperature contrast with their background (Schraft et al., 2018; Shine et al., 2002). Notably, a patterned thermal background may impair predatory target detection. Sidewinders (*C. cerastes*, Schraft et al., 2019) and Chinese Pit-Vipers (*Gloydius shedaoensis*, Shine and Sun, 2002) choosing ambush sites appear to orient toward the background with the least structure, suggesting that background temperature patterning could interfere with effective predation. This would be expected if the neural image has poor resolution.

However, other behaviors suggest better resolution. Specifically, the body temperatures of small mammals change during antipredator interactions with rattlesnakes, perhaps due to elevated metabolism related to flight-or-fight responses (Cabanac and Briese, 1992; Southwick, 1973). Resulting surface temperatures show interesting patterns. California ground squirrels have higher temperatures in the tail region when interacting with Western Rattlesnakes (*Crotalus oreganus*) (Rundus et al., 2007), and Merriam's kangaroo rats show increased head, snout, and hind leg temperature after detecting Mojave Rattlesnakes (*C. scutulatus*) (Schraft and Clark, 2017; Schraft et al., 2018). In both cases the degree of temperature change was relatively minor and localized to a particular part of the body. While likely related to the use of appendages to dissipate metabolic (e.g., Conley and Porter, 1985), signaling functions have been suggested with some experimental support (Rundus et al., 2007). Although such changes are readily detected with a thermal imaging camera, it remains to be demonstrated that the pit organ system could resolve those details.

Behavioral experiments can evaluate the neural image informing behavior, and thus assay the overall performance of a sensory system. Laboratory studies of facial pit function done to date have presented pitvipers with both static and moving thermal targets that strongly contrasted with a uniform thermal background (e.g., Bakken et al., 2018; Chen et al., 2017; Cock Buning et al., 1981; Ebert and Westhoff, 2006; Krochmal and Bakken, 2003; Van Dyke and Grace, 2010). While these experiments maximize the potential for pitvipers to detect target stimuli and assay sensitivity to thermal contrast, they provide little information about angular resolution.

Our study therefore addressed the resolution of the neural image informing behavior with an evoked behavior assay of facial pit spatial resolution. We designed an apparatus and experiment that could allow us to use behavioral responses to create a thermal image contrast sensitivity function (CSF) plot. The CSF is a plot of contrast sensitivity of an imaging system as a function of the spatial frequency

of the source (units 1/deg). Vision studies typically record neural or behavioral responses to bar or checkerboard stimuli with defined spatial frequencies to compare the visual performance of different animal species (reviewed by da Silva Souza et al., 2011). We used the Western Rattlesnake, *Crotalus oreganus*, as our study species as it has been used extensively in facial pit studies that provide context and supporting information for our study (Bakken et al., 2018; Berson and Hartline, 1988; Hartline et al., 1978; Haverly and Kardong, 1996; Kardong, 1992; Newman and Hartline, 1981; Rundus et al., 2007; Stanford and Hartline, 1984). Importantly, this species was used in a study that determined the PSF as represented in the LTTD (Stanford and Hartline, 1980). It is known that this species further processes the pit image in higher brain centers and merges it with visual information in the optic tectum (Berson and Hartline, 1988; Newman and Hartline, 1981).

Our experiment was designed to test the hypothesis that processing in higher centers (RC, optic tectum, and telencephalon) may further improve resolution. Our null hypothesis was based on the degree of image sharpening demonstrated in the LTTD (Stanford and Hartline, 1984). If the CSF determined in our apparatus indicated a higher spatial frequency response than the ca. 0.1 deg^{-1} demonstrated in the LTTD (Fig. 6, Stanford and Hartline, 1984), we could reject the null hypothesis and suggest that additional image sharpening occurs in the RC, optic tectum, and/or telencephalon of *C. oreganus*. To test this hypothesis, we modified a laboratory-based apparatus that reliably elicited snake responses to thermal targets in a previous study (Bakken et al., 2018). We added perforated metal grids to the apparatus to create patterned thermal backgrounds behind a moving pendulum with a temperature regulated bob. Our apparatus design was based on the prediction that a target with a uniform surface temperature moving over a uniformly patterned thermal background with the same average temperature would release a response from a pitviper only if the background pattern could be detected. Else, the scene would appear to have a uniform temperature identical to the target temperature, and the target would thus be invisible. This is the same principle used by computer displays, where a pattern of colored dots, individually too small to be resolved by human eyes, creates the appearance of a continuous shaded surface. Thus, if the background was detected, we predicted the response would be stronger to targets moving across coarser or higher temperature contrast thermal grids, we could use this relationship to define a CSF for the pit organ system. Knowledge of the PSF or CSF can be used to simulate the snake's thermal image of any scene recorded by a thermal camera (Bakken and Krochmal, 2007). The pattern dimensions were selected to test for a response to higher spatial frequencies that would indicate image sharpening beyond that established for the LTTD, ca. 0.1 deg^{-1}.

MATERIALS AND METHODS

Study subjects

Experimental subjects were 17 adult Western Rattlesnakes (*Crotalus oreganus*) that were long-term captives in the vivarium at San Diego State University. Individuals had been in captivity for varying periods, but all for longer than a year. Twelve individuals were "problem" snakes that were being translocated away from populated areas of Camp Pendleton Marine Corps Base in San Diego, CA, and 5 had been collected from other areas of San Diego County (California Fish and Wildlife Scientific Collecting Permit SC-9704). Animals were housed in $60 \times 40 \times 40$ cm plastic terraria with paper substrate, and provided water ad libitum. Each terrarium had a wooden hide box that snakes used as a retreat site. This box had a sliding door that could be closed, and thereby used to transport snakes to and from the experimental apparatus with no direct handling. Procedures were approved by the SDSU IACUC protocol APF 19-08-009C.

Experimental apparatus

We used a modification of the experimental apparatus described in Bakken et al. (2018). In brief, the apparatus consisted of a 61×41 cm rectangular temperature-controlled backing plate that provided a thermally homogenous surface. A perforated plate was mounted 3 cm in front. This distance was great enough that the perforated plate remained near air temperature. Together, these established a 2-temperature patterned background with defined contrast and spatial frequency. By adjusting the temperature of the background plate relative to air temperature, we could establish specific background pattern temperature contrasts (Fig. 1).

Each perforated plate consisted of a of 2 mm thick aluminum sheet perforated with a hexagonal pattern of regularly spaced circular holes to create a grid with a defined spatial frequency approximating the checkerboard stimulus used in visual studies. These had a 1:1 ratio of metal to open space, such that the average background temperature was the average of the temperatures of the backing and perforated plates (Fig. 1). We used six plates, each with 8, 13, 19, 25, 32, and 38mm diameter perforations in a hexagonal pattern with a center-to-center distance of $1.347 \times$ diameter. Although we conducted experiments in complete darkness to eliminate the possibility of visual stimuli, the background plate, the perforated plate, and the pendulum bob were all painted a uniform flat black (thermal emissivity ca. 0.98; 1916 Ultra-Flat Black, Rust-Oleum, Vernon Hills, IL, USA) to further reduce the possibility of visual stimuli, and also ensure emitted thermal radiation was accurately related to temperature.

To measure background pattern temperatures, 4 thermocouples were embedded in the backing plate. Each perforated plate also had 4 thermocouples, cemented to the back using steel-filled epoxy resin (JB Weld Steelstik 8267-S). Both sets of 4 thermocouples were connected in an equal-arm "spider" pattern that sensed average temperatures with 0.1°C resolution. Temperatures were recorded by a digital data logger (CR23X, Campbell Scientific, Logan, UT, USA). Temperature uniformity was verified by thermography.

The stimulus target was a pendulum bob suspended 3 cm in front of the perforated plate. It consisted of a hexagonal aluminum plate (7.0 cm across the flats × 0.63 cm thick) with a square Peltier element (5 x 5 cm; model 12711-9M31-24CW, Custom Thermoelectric, Bishopville, MD, USA) and heat sink cemented to the back. It was suspended from 2 - 1 m lengths of 2.6 mm o.d. hypodermic tubing, each containing 1 of the 0.8 mm conductor wires supplying power to the Peltier element. Each tube also contained a Teflon insulated duplex thermocouple with 0.013 mm diameter conductors (type TT, Omega, Norwalk, CT, USA). The sensing tips of these thermocouples were electrically insulated and inserted in close-fitting holes drilled 1cm deep into the edge of the bob. One thermocouple was connected to a custom electronic circuit that controlled the temperature of the bob, and the other monitored bob temperature.

The temperature of the stimulus pendulum bob and background elements were recorded by the datalogger. Datalogger support software (PC 200W 4.4.2, Campbell Scientific, Logan, UT, USA) computed and displayed the average background temperature, the pendulum bob temperature, and their difference in real time at the operator station. The operator could thereby adjust the temperature of the pendulum bob to reduce the difference from average background temperature to 0.3°C or less. We thereby presented test subjects with a moving pendulum bob that had essentially the same temperature as the average temperature of the patterned background it moved across (Fig. 1).

The procedure room in which the tests were conducted was kept between 20–22°C, and snakes were housed in this room for at least 4 hours prior to testing so that their body temperature conformed to the room temperature. This temperature range is both ecologically realistic (many pitvipers in temperate climates are nocturnal, and ambush small mammals and other endothermic prey through the cooler nighttime periods). Also, this corresponds to the body temperature at which we found the *C. oreganus* in our previous experiment to be most responsive to our thermal stimuli (Bakken et al., 2018). We did not test snakes that had eaten within the past week, so that test subjects would not be satiated and would be more likely to respond to relatively small moving thermal stimuli that might represent prey.

For each test, the sliding door was inserted in the hide box containing the rattlesnake and transported from the home cage to the procedure room (an adjacent room with no lights), where it remained for at least 4 hours to allow the body temperature of the snake to equilibrate with the ambient temperature of the room. Prior to test, the hide box was placed on a pedestal facing the testing apparatus with the front of the hide box 30 cm from the perforated plate. The sliding door was attached to a string running through a long, curved copper tube to the operator's station. We could then raise and lower the hide box door remotely to reveal the testing apparatus to the snake. The curve in the copper tube blocked light from the operator's station in the control room. The pendulum bob was held to the side by an electromagnet. When the electromagnet was turned off, the bob was released silently and swung back and forth in front of the perforated plate. A recording video camera using invisible near-IR illumination allowed us to view the snake at the time of pendulum release and record responses. See Bakken et al. (2018) for further details on the basic design and construction of the apparatus.

Experimental design

The operation of our experiment is illustrated in a video (Video S1) of a stimulus presentation recorded with a thermal imaging camera (FLIR model T420, FLIR Systems, Inc., Arlington, VA USA). The video shows the pendulum bob moving in front of the metal plate with 8 mm circular perforations, with the pendulum bob set to the average temperature of the cool and warm portions of the background. When the camera is defocused, the background is not resolved and the pendulum cannot easily be perceived, but when the camera is refocused so the gridded background is resolved, the bob is seen as a distinct moving object.

Comparative analyses of visual acuity in vertebrates typically present test subjects with a video display of alternating or moving dark and light gratings or checkerboard patterns with spatial frequencies and contrasts as variables. The responses of the animal (eye or head movements, neurophysiological evoked potentials, etc.) indicate when stimuli were perceived by the study subject (e.g., Baker et al., 2007; Keller et al., 2000; Schmid and Wildsoet, 1998; Tappeiner et al., 2012). The data are presented as a contrast sensitivity function (CSF), a plot of response vs. logarithm of spatial frequency (reviewed by da Silva Souza et al., 2011). The highest spatial frequency that the animal responds to is used as a measure of visual acuity.

We approximated the checkerboard stimulus with grids consisting of perforated plates with center-to-center angular distances between perforations of $2 \times \tan^{-1}[(1.347 \times \text{diameter})/(2 \times \text{distance to perforated plate})]$. The lowest (and thus most detectable) spatial frequency of the pattern is the reciprocal

of this angular distance. For our largest (38mm) perforation at ca. 32–33 cm from the snake's pits, the angular distance is ca. 9° and the spatial frequency is ca. 0.11 deg^{-1}, approximately equal to that inferred from the best (forward direction) PSF recorded by Stanford and Hartline (1984).

We used a cyclical permutation of different stimulus presentations to create a balanced order of contrast values and perforation sizes presented to the 17 rattlesnakes used. Each was tested with all 6 metal grids, with the order of trials balanced across study subjects such that either 3 or occasionally 2 individuals started the grid sequence at each of the 6 perforation sizes. A minimum of 3 weeks elapsed between subsequent trials for the same individual. During each trial, the snake was shown the pendulum moving across1gridded background with a series of 6 different temperature contrasts (difference between cool and warm portions of background). The metal grid conformed closely to the air temperature of the room, 20–22°C. Backing plate temperature was varied using a temperature-controlled water bath (VWR Scientific model 1160, Chicago, IL, USA) to establish background contrast values of approximately 0°, 3°, 6°, 9°, 12°, and 15°C. As with the perforation sizes, the order of contrast values was balanced so that either 2 or 3 individuals began the sequence at each of the six contrast values. In order to alter the contrast value between each pendulum presentation, the opaque door of the box containing the test subject was lowered and a researcher entered the procedure room to adjust the temperature of the water bath heating the background plate and reattach the pendulum to the electromagnet. The pendulum temperature was then readjusted so that at the time of release it matched (within 0.3°C) the average temperature of the metal perforation and background plate. Between 5–30 minutes elapsed between presentations, depending on the time necessary for the snake to cease spontaneous activity and for the pendulum to either heat or cool to the next target contrast value.

Using this design, all 17 snakes were tested with all six background perforations, and during each test all six contrast values were presented, resulting in a total of 612 stimulus presentations. To minimize habituation, we rotated through all 17 experimental subjects, leading to a minimum 3-week period between subsequent trials for an individual snake. This procedure successfully eliminated habituation in a previous study (Bakken et al., 2018). Given the large number of stimulus presentations, we also performed a post-study habituation control trial with the perforated metal plate removed so the background was uniform. This established the same experimental condition as in Bakken et al. (2018), so that it could be used as a reference for responsiveness of test subjects in our apparatus.

Behavioral scoring and statistical analysis

As in our previous experiment (Bakken et al., 2018), we used the evoked behavioral response of the snakes in the form of tongue flicks and head movements to score responses to stimuli. Once snakes were positioned in the apparatus, they were allowed to settle for at least 3 minutes before any test was conducted. We also did not release the pendulum stimulus in any trial until the snake had exhibited at least 30 seconds of complete stillness (no movements or tongue flicks).

After releasing the pendulum stimulus, we scored behaviors during the 5 s following the pendulum release, corresponding to the first 3 swings of the pendulum. A positive response was scored if any tongue flicks (TF) or head movements (HM) were exhibited during this 5 s period, and a negative response if no overt movements were exhibited. To quantify tongue flicks and head movement response strength, we counted the total number of video frames (out of 150) where the tongue was extruded or the head had changed position from the previous frame. Thus, each pendulum presentation resulted in 3 response variables: (1) yes/no response to stimulus, (2) TF score, and (3) HM score.

We examined these 3 response variables independently using a generalized linear mixed model (GLMM) framework. Because our TF and HM scores are zero-inflated count data, we used negative binomial distributions for these responses, and a binomial distribution for the yes/no response data. We included the diameter of perforations, the contrast between warm and cool pattern elements, and their interaction as fixed factors. We included a random intercept for snake ID to account for repeated measures on the same individuals. Because we designed the experiment to balance trial order and stimulus order in a permuted fashion, we did not include these as factors in the model, but we did visualize them to see if there was any apparent association with our response variables. All analyses were performed in R versions 4.0.5 using the package glmmTMB for mixed model analysis. The raw data are available as supplementary material (Dataset 1).

To aid our interpretation of the results, we used GNU Octave 5.1.0.0 (Eaton et al., 2020) to simulate an expected contrast sensitivity function (da Silva Souza et al., 2011) assuming a disk spread function of 10° angular diameter using the methods of Bakken and Krochmal (2007). The 10° diameter is based on the best neurophysiological result (recorded directly in front of the snake) found by Stanford and Hartline (1980) in the LTTD of snakes of the same study species as ours. The contrast response function is modeled as the ratio of the background pattern contrast in the image to that in the source scene (Fig. 2). Sensitivity functions were modeled assuming (1) the standard sinusoidal background used in visual experiments, (2) the hexagonal perforated plate geometry used in our experiment, and finally (3) the average over 27 positions of1simulated swing of the hexagonal pendulum over the perforated plate. The

spatial frequencies and perforation diameters of our plates are indicated by labeled vertical lines. If Stanford and Hartline (1984) adequately described the final neural image, these functions lead us to expect a weak response to the largest perforations, and no response to the smaller perforations. A response to the higher angular frequencies of the smaller perforations would provide evidence for additional overall neural image sharpening beyond the LTTD, as well as the data needed to generate a CSF describing any such enhanced facial pit imaging. A lack of response would indicate no further sharpening beyond the LTTD, with functional angular resolution perhaps no better than the 20° found for other view angles (Stanford and Hartline, 1980,1984).

RESULTS

Snakes in trials with patterned backgrounds exhibited very limited responsiveness to the pendulum stimulus, regardless of perforation size or the degree of contrast between the target and cool portions of the grid (Fig. 3). The tongue-flick response model indicated that snake responsiveness was not affected by perforation size, contrast, or the interaction between perforation size and contrast (Fig. 3, Table 1). Similar results were obtained for the head movement and binary response models (Table 1). Even at the highest levels of contrast and largest perforation sizes, snakes exhibited similar background responsiveness to all levels of target contrast.

Our post-experiment control test showed that the tested snakes had not habituated to the testing apparatus, as the same snakes responded strongly to the pendulum stimulus when presented with a homogenous thermal background after the main study. Although small differences in experimental design prevent statistical comparisons, the response of snakes in the post-experiment control trial were similar in magnitude to the responses exhibited by snakes in Bakken et al. (2018), where the same species and apparatus and similar testing conditions were used (Fig. 3).

DISCUSSION

Sensory performance

Although the pit organ aids prey detection and targeting (Goris, 2011), as well as locating thermal refuges (Krochmal and Bakken, 2003), our experimental results provided no evidence that the neural thermal image formed by *C. oreganus* can resolve anything with an angular extent less than or equal to the ca. 9° – 10° (spatial frequency ca. 0.1 deg^{-1}) indicated for the forward direction by figure 6 in Stanford and

Hartline (1984). The range of higher spatial frequencies ($0.11 - 0.54$ deg^{-1}) covered in our study did not allow us to estimate either the threshold spatial frequency at which our snakes could actually resolve spatial details, or generate a measured CSF to replace our prediction (Fig. 3) based on Stanford and Hartline's (1980, 1984) work. Thus, we cannot reject our null hypothesis that, for *C. oreganus*, thermal image sharpening occurs only in the LTTD.

Our results for *C. oreganus* may not apply precisely to other species, as Bakken et al. (2012) found significant variation among the pit apertures of specimens of 4 species, with the *C. atrox* specimen having a significantly smaller relative aperture, and hence better resolution on the facial pit membrane, than the *C. oreganus specimen*. Conversely, *C. horridus* and *Cryptelytrops albolabris* specimens had a larger aperture. These differences may or may not carry through neural processing to the neural image informing behavior. Therefore, we conclude that it is important to repeat our study on other species before generalizing.

Clearly, it is not accurate to use high-resolution images and videos as recorded by commercial thermal imagers to visualize the thermal radiation image that a pitviper might perceive (e.g., in den Bosch, 1983; Kardong, 1986; Rundus et al., 2007). However, further image processing as has been done for the pit membrane image may provide useful visualizations (Bakken et al., 2012; Bakken and Krochmal, 2007). For an example of computed thermal imaging of a natural scene based on the neural PSF derived from Stanford and Hartline (1984), see Schraft et al. (2019).

As an assay of the overall performance of thermal imaging, including neural processing, our experimental results redirect attention to the question of how thermal and visual images with radically different angular resolutions are merged in the optic tectum, and how the results are presented to the telencephalon to inform behavior. The presence of neurons responding to both visual and infrared inputs provide the primary evidence for a multispectral visual image (Goris, 2011; Gower et al., 2019; Hartline et al., 1978; Newman and Hartline, 1981). However, the spatiotopic maps of the receptive fields for thermal and visual stimuli were poorly aligned, which argued against a multispectral neural image (Hartline et al., 1978). Together with our results, it is not clear that there is a basis for postulating a multispectral neural image in the telencephalon, let alone one with angular resolution of thermal imagers.

Newman and Hartline (1981) reviewed studies of many species demonstrating that other low resolution spatial inputs (auditory, somatosensory) were merged with visual information in their tecta with similar misalignment of the spatiotopic maps. They suggested that the low-resolution spatial information, rather than forming a multispectral image, might serve as a feature detector by directing attention to visual inputs with certain characteristics, such as a moving warm animal vs. a windblown leaf.

Synergistic interaction of visible and thermal input is suggested by the reduced strike range when only thermal input is available (cf. Table 1, Kardong, 1992).

We conclude that behavioral research is needed on all aspects of the integration of thermal and visual information. Neurophysiological (Hartline et al., 1978) and behavioral (Chen et al., 2012) studies suggest that the pit organ functions best as a part of an integrated system, but our tests and similar studies by others (e.g., Chen et al., 2012; Ebert and Westhoff, 2006; Kardong, 1992) have all been conducted with the visual system disabled by darkness or blindfolds — conditions which isolate the thermal sense, but block the ability to integrate visual and thermal stimuli.

Ecology

A completely dark testing environment is, in fact, not representative of the typical ecological setting for ambush-hunting pit vipers. The limited research into snake visual systems indicate that they are adapted for low light environments, similar to other nocturnal predators (Baker et al., 2007; Bittencourta et al., 2019; Simoes et al., 2016). It is likely that even when hunting at night, under deep forest canopies, or heavy cloud cover, there is enough light for visual stimuli to be salient. Foraging in complete darkness would occur but rarely. Therefore, it is possible that synergy between visual and thermal stimuli occurs in ecological situations where both stimuli are present at submaximal levels.

Most studies of pit organ function have been conducted in laboratory environments, in arenas that lack the thermal heterogeneity common to field settings. Background features may result in the thermal representation of a prey animal displaced from its actual location (Fig. 4B, Bakken and Krochmal, 2007) and (Figs. 4 C, 4 F, Bakken et al., 2012). Our results further indicate that a prey item may even not be visible in a thermal radiation image with a complex background. Indeed, field studies (Schraft et al., 2019; Shine and Sun, 2002) indicate snakes in ambush posture avoid thermally heterogenous backgrounds, suggesting that complex thermal backgrounds impair prey targeting.

Provided that the target can be isolated against its background, lack of detail does not necessarily impair targeting, as the center of a large, diffuse, poorly resolved object can be located surprisingly accurately (Kontsevich and Tyler, 1998; Yovel et al., 2010). Thus, targeting prey and locating thermal refuges is feasible even with low resolution (Krochmal and Bakken, 2003). Some studies suggesting detailed imaging used uniform thermal backgrounds, and thus are actually consistent with our results. Kardong (1986) found that *C. oreganus* struck preferentially at the head of mice to avoid retaliatory bites, but with pits blocked, the range at which they would strike was but 5 cm (Kardong, 1992). At this range, with a ca. 1.5 cm long head, the angle subtended is ca. $\tan^{-1}(1.5/5) = 16°$ (0.06 deg^{-1}). Similarly, Rundus et

al. (2007) found that California ground squirrels, *Otospermophilus (Spermophilus) beecheyi,* vasodilate their 13 cm – 23 cm long tail and flag at rattlesnakes which respond, suggesting decent resolution. However, the definitive test used a uniform background with a life-size taxidermic model placed 40 cm from the snake. The tail thus presented a target that subtended 18° to 30° degrees (0.6 to 0.3 deg^{-1}). Further, motion by a thermal radiation source increases neural response (Hartline et al., 1978). Even though the temperature contrast was only 2°C, the tail stimulus is equivalent to a pendulum stimulus that produced a ca. 30% of maximum response in Bakken et al. (2018).

However, given complex thermal backgrounds in natural habitats, pitvipers may be unable to resolve sufficient spatial detail to perceive the thermographically recorded regional body temperature changes of smaller prey at greater distances. In the field, snakes may launch predatory strikes at distances of 40-50 cm (pers. obs.). This would change the angle subtended by the head of a mouse to 1.7° - 2.1° (0.6 – 0.5 deg^{-1}), much less than the 16° in situations where snakes struck using only pit inputs in the study by Kardong (1992). Snakes might strike at the strongest signal, but with background interference the center of the strongest signal may not coincide with the head (Bakken et al., 2012; Bakken and Krochmal, 2007). Regional temperature differences in prey, such as those documented in Schraft and Clark (2017), would typically be too minor to be distinguished.

Evolution

The evolutionary origin of the pitviper facial pit is essentially unknown. The oldest viper fossils are not significantly different from modern forms, and the fossil record of pitvipers is effectively absent in the south-southeast Asian area of origination (Szyndlar and Rage, 2002). In any event, fossils only rarely provide evidence for physiological or ecological selection processes. Thus, we are left to speculate using general principles.

The neuroanatomical evidence for visual and thermal radiation merger have implied a multispectral neural image with decent resolution (Berson and Hartline, 1988; Newman et al., 1980). Gower et al. (2019) compared visual systems of vipers with and without pits, and argued that thermal radiation imaging evolved as an elaboration of the existing visual system.

However, the behavioral results of this study support pit membrane image modeling that indicated pit organs are not able to resolve detailed spatial pattern and most effectively detect objects with substantial temperature contrast moving across homogenous thermal backgrounds. (Bakken et al., 2012; Bakken and Krochmal, 2007). While neural image sharpening improves imaging somewhat, our results show the improvement is not sufficient to alter these conclusions.

Krochmal et al. (2004) and Bakken and Krochmal (2007) speculated that the ancestral facial pit was comparatively insensitive to receptor temperature change and had little angular resolution. The sense would possibly arise in a habitat with strong temperature contrasts. Selection might favor a relatively insensitive system using a simple directional mechanism such as null detection with receptors on opposite sides of the face. They suggested several scenarios with strong thermal contrast and minimal resolution requirements. First, the ability to sense thermal radiation indicating substantial habitat temperature differences might aid thermoregulation, for example by avoiding dangerously sun heated patches on a dark substrate that provides limited visual indication of the strength of incident sunlight, or by finding warm nocturnal basking sites. Second, the ancestral pitviper may have been arboreal, feeding on endothermic birds or bats that contrasted strongly with a cold sky background (Shine and Sun, 2002; Swinbank, 1963). Third, endothermic prey might be detected by a snake searching cool burrows with little demand for ability to detect signal direction. Fourth, an approaching larger predator would provide an extended, and thus stronger, signal.

We can only add the possibility that most extant pitvipers are ambush hunters that generally lie in wait for prey for very long periods of time without moving (reviewed in Beaupre and Montgomery, 2007), and so ancestral pitvipers likely also relied on ambush foraging. Perhaps the pit organ operated as an "alarm" system that has or had a lower threshold of activation from stillness (or even sleep) than other sensory systems. Thus, it could serve to arouse an inactive snake and bring the attention of vision to bear when some low-resolution aspect of the thermal radiation field is changing, as when endothermic prey or predators move nearby.

Improving the use of thermal infrared information to inform behavior is a likely feature of the continuing evolution of the thermal radiation sense. Krochmal et al. (2018) provide a possible example. After only one trial, rattlesnakes (*Crotalus* and *Sistrurus*) of 7 different species quickly identified and moved toward a cool refuge within a stressful hot environment by sensing thermal radiation alone. All 6 species of non-rattlesnake pit vipers tested took 5 times as long to move to the cool refuge, and failed to modify their behavior across 12 trials. This absence of learning was also observed in the true viper, *Bitis arietans*. As rattlesnakes are the most derived viper clade, this suggests continuing evolution of more effective utilization of thermal infrared information.

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Author contributions

Conceptualization: G.S.B., R.W.C.; Methodology: G.S.B., R.W.C.; Validation: R.W.C.; Formal analysis: R.W.C.; Investigation: R.W.C., E.J.R., A.S.; Resources: G.S.B., R.W.C.; Data curation: R.W. C.; Writing - original draft: R.W.C., G.S.B.; Supervision: R.W.C.; Project administration: R.W.C., G.S.B.

Supplemental materials

Video S1. Video recording of testing apparatus made with a thermal imaging camera (FLIR model T420, FLIR Systems, Inc., Arlington, VA USA). When the camera is defocused, the background is not resolved and the pendulum cannot easily be perceived, but when the camera is focused so the gridded background is resolved, the pendulum is seen as a distinct moving object.

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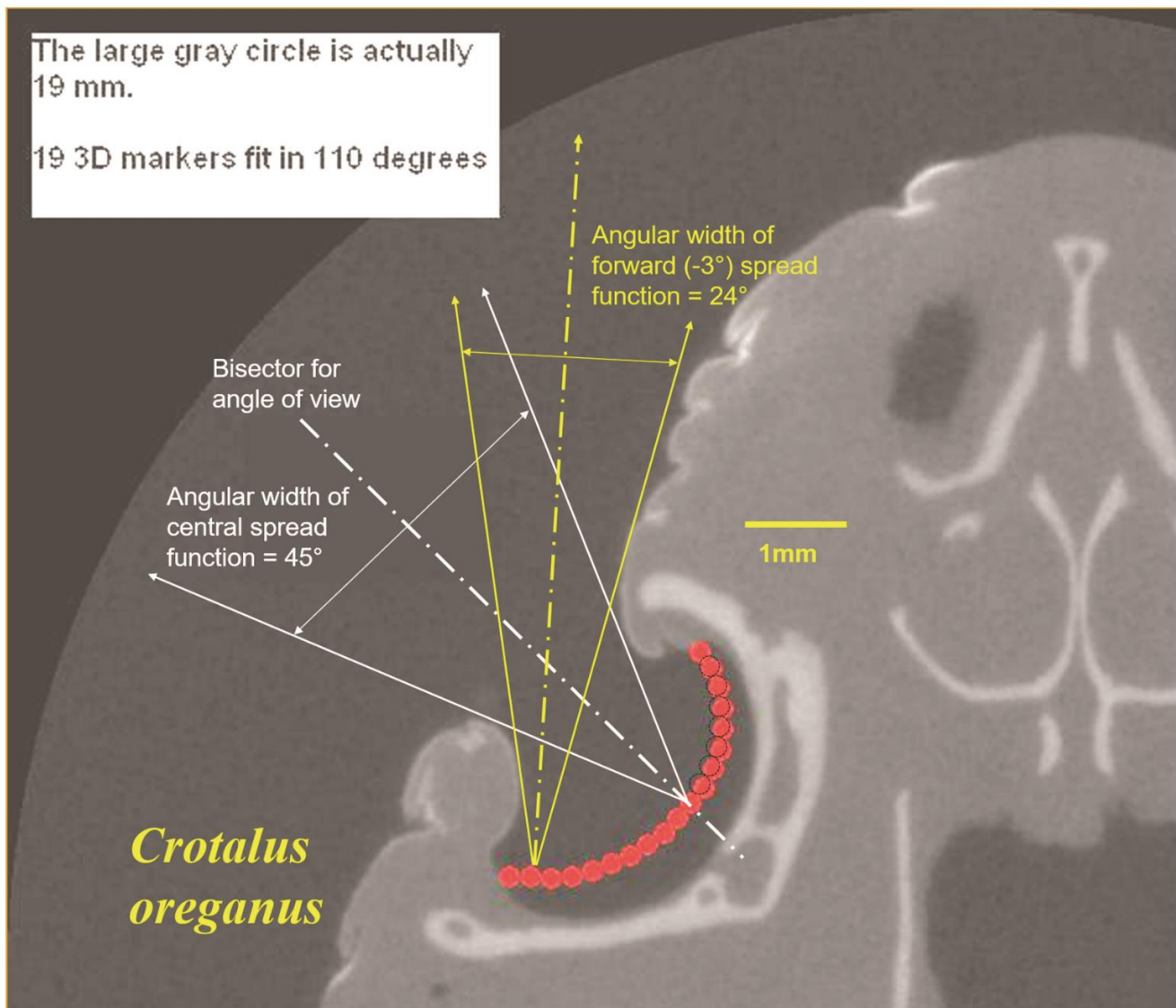
Figures and Table

Fig. 1. Geometric spread functions for *Crotalus oreganus*. This X-ray tomography view of the nose and facial pit has been marked to indicate the view angle of a single sensory ending on the pit membrane corresponding to the angular neuron response in the LTTD in Fig. 6 of Stanford and Hartline (1984). The angular width of the neural response field in the forward (-3°) direction was ca. 10°, vs. 24° for the geometric view angle in this image, indicating ca. 2X neural sharpening. Similarly, the typical lateral neural response field was ca. 20°, again ca. half that of the lateral geometric view angle in this image. The red markers indicate the position of the sensory membrane suspended in the pit. This section is from the same *C. oreganus* specimen used by Bakken et al. (2012).
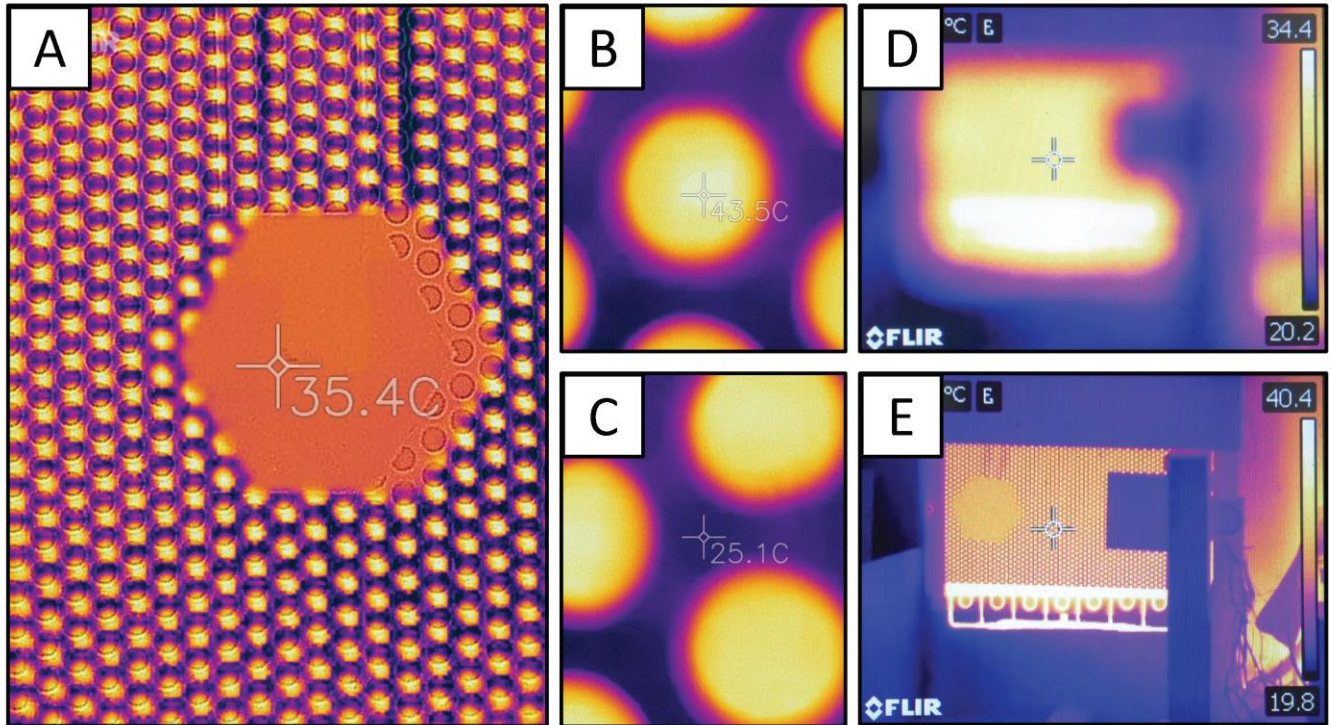
Fig. 2. Thermal images demonstrating experimental set up. The hexagonal pendulum target (A) in front of a patterned thermal background. Thermal background (A) was made by placing a metal sheet with 8 mm circular perforations in a gridded pattern in front of a warmed epoxy plate. Metal plates had different sized perforations, but all created a thermal background with a 1:1 ratio of warm to cool areas. Pendulum target temperatures were set so that they were always at the average temperature of the warm (B) and cool (C) portions of the background. An imaging system that cannot resolve the spatial details of the background plate, such as a defocused thermal imaging camera (D), does not resolve a target that is distinct from the background. A system that can resolve the background, such as a focused thermal imaging camera (E), can resolve the target pendulum. See supplementary video 1 for videographic demonstration of pendulum moving across grid with focused and defocused imagery.
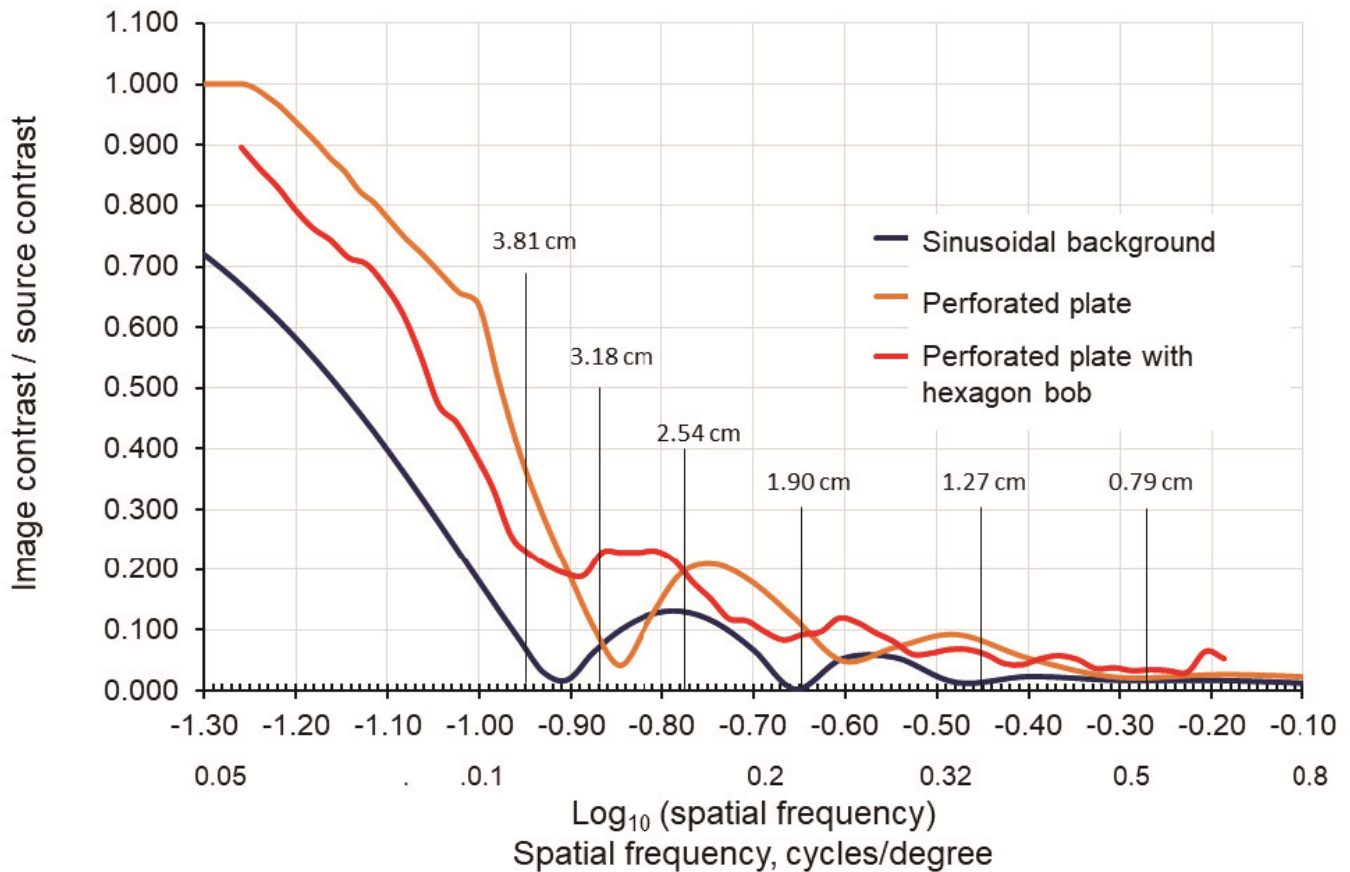
**Fig. 3. Reference hypothetical contrast sensitivity function assuming a disk spread function of 10°
angular diameter vs. the fundamental spatial frequency of different sources.** The response function is
modeled as the ratio of the contrast in the image to the contrast in the source scene. Results are shown for
(blue) a standard sinusoidal background as used in visual experiments, (orange) the hexagonal perforated
plate geometry used in our experiment, and (red) the average over 27 positions of one simulated swing of
our hexagonal pendulum bob moving over the perforated plate. The 10° disc spread function is based on
the best neurophysiological result obtained by Stanford and Hartline (1984). The perforated plate predicts
CSF a stronger but variable response at higher spatial frequencies than a pure sinusoidal source, but for all
backgrounds the indicated resolution found by extrapolating the curves from ca. 0.6-0.3 to 0 is
approximately the same (spatial frequency ca. 0.12 deg^{-1}). The spatial frequencies and perforation
diameters of our plates are indicated by labeled vertical lines. The detectability of aliased patterns appears
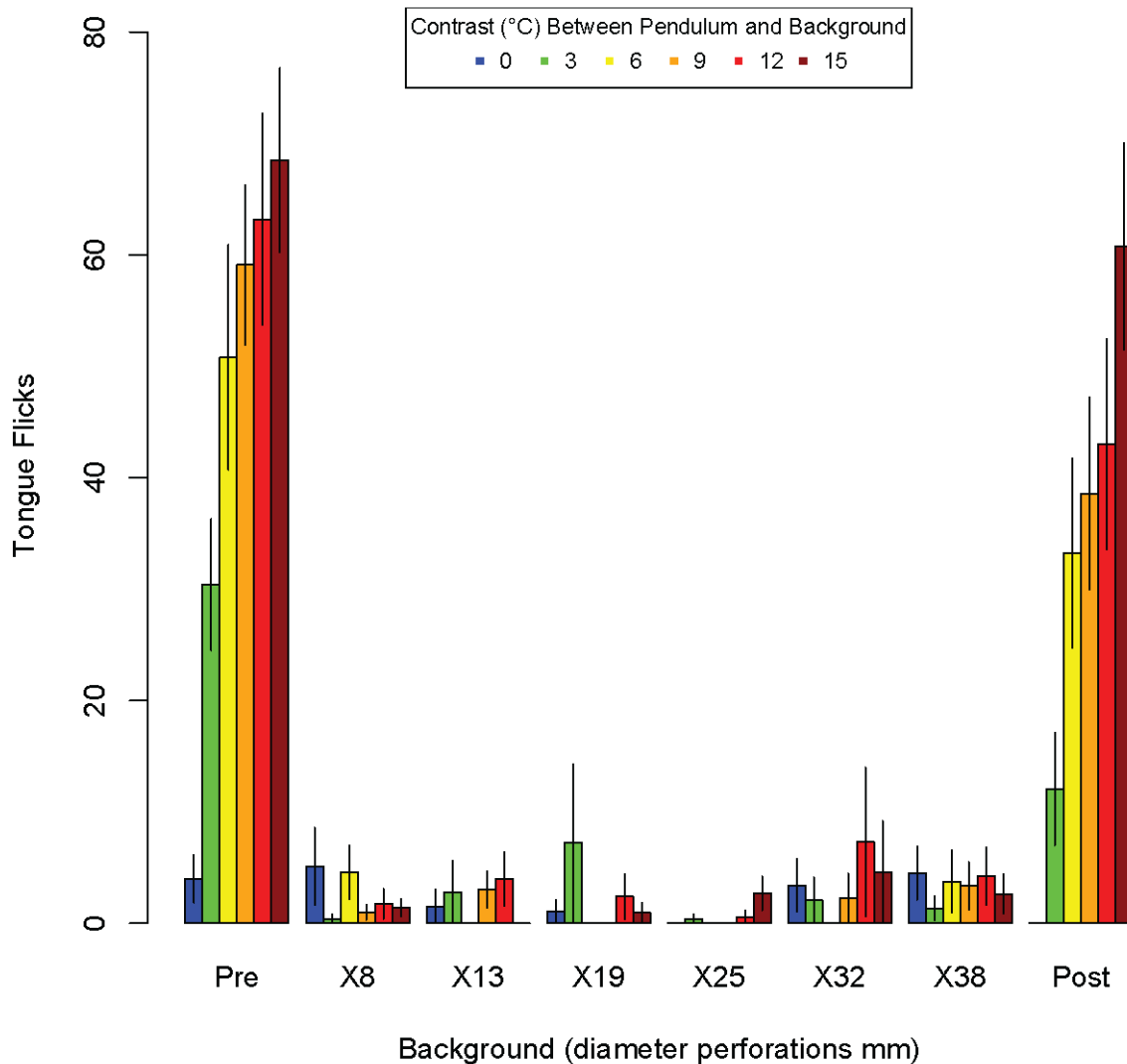as oscillations at higher spatial frequencies.

Fig. 4. Tongue flick responses to thermal targets presented to snakes across a range of background patterns (size of perforations in metal plates) and contrast values. Histograms indicate mean ± standard error of number of video frames in which tongue was extruded (TF scores). There is no evidence of a significant response to the moving pendulum for any contrast or perforation diameter. Habituation due to repeated use of each animal in the long series of experiments might account for this non-response. The figure includes post-experiment results on the right with the grid removed, showing snakes still retain adequate responsiveness to stimulus when the thermal background is homogenous ("Post"). The responsiveness of snakes in an earlier study (Bakken et al., 2018) using the same apparatus with no background plate is shown at left for comparison ("Pre"). *N* = 612 trials on 17 snakes.

Table 1. Results from models of tongue-flick response (number of 150 frames in which tongue was extruded), head movement response (number of 150 frames in which head had moved from position in previous frame), and binary response (yes/no for if a snake responded to a target presentation) as a function of target contrast, perforation size, and the interaction between contrast and perforation size. All models included a random intercept for snake identity. N = 612 trials on 17 snakes.

	Estimate	SE	z-value	*p*-value
Tongue flick response				
Intercept	0.69	1.09	0.63	0.53
Contrast	-0.07	0.11	-0.63	0.53
Perforation size	-0.18	1.03	-0.17	0.87
Contrast:Perforation	0.05	0.11	0.52	0.60
Head movement response				
Intercept	-5.82	3.23	-1.80	0.07
Contrast	-0.22	0.25	-0.87	0.38
Perforation size	-7.94	4.43	-1.79	0.07
Contrast: Perforation	0.59	0.38	1.55	0.12
Binary response				
Intercept	-3.38	0.71	-4.73	0.00
Contrast	0.07	0.07	1.04	0.30
Perforation size	0.63	0.64	0.99	0.32
Contrast: Perforation	-0.05	0.07	-0.67	0.50
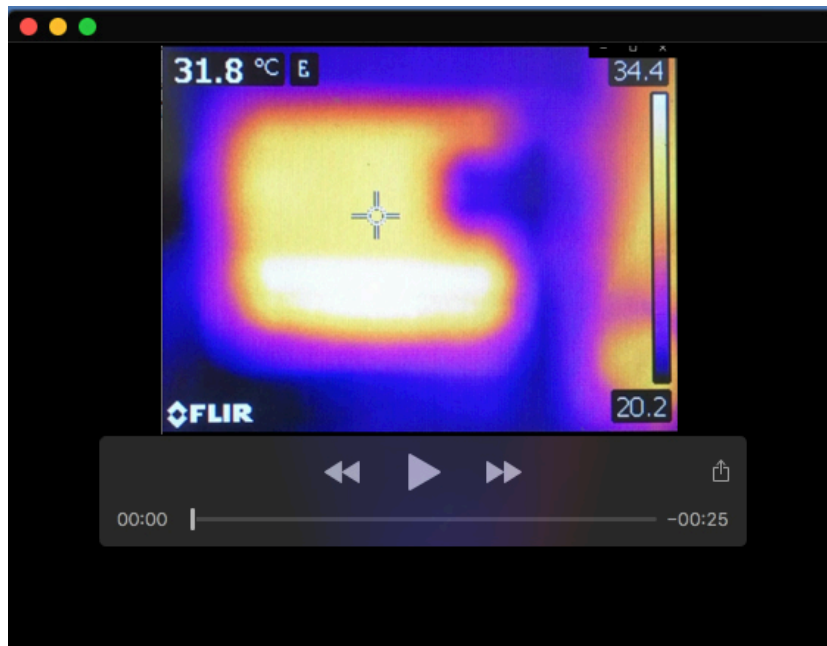
Movie 1. Video illustrating the principle of the experimental setup using a defocused thermal imaging camera. When the camera is defocused at the start of the video, the background plate is not resolved by the imaging system and the moving pendulum target cannot be distinguished from the background because it is the same temperature as the average background temperature. When the camera is focused, the patterning of the background is resolved as a series of warmer and cooler areas, and the moving pendulum target is seen more clearly.