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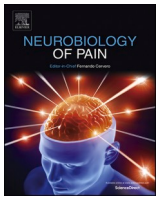

Original Research

# Thermal escape box: A cost-benefit evaluation paradigm for investigating thermosensation and thermal pain

Jacquelyn R. Dayton [a,1], Jose Marquez [a,1], Alejandra K. Romo [a], Yi-Je Chen [b], Jorge E. Contreras [a], Theanne N. Griffith [a,*]

[a] University of California, Davis. Department of Physiology & Membrane Biology, 1275 Med Science Drive, Davis, CA 95616, United States
[b] University of California, Davis. Department of Pharmacology, 1275 Med Science Drive, Davis, CA 95616, United States

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ABSTRACT

Thermosensation, the ability to detect and estimate temperature, is an evolutionarily conserved process that is essential for survival. Thermosensing is impaired in various pain syndromes, resulting in thermal allodynia, the perception of an innocuous temperature as painful, or thermal hyperalgesia, an exacerbated perception of a painful thermal stimulus. Several behavioral assays exist to study thermosensation and thermal pain in rodents, however, most rely on reflexive withdrawal responses or the subjective quantification of spontaneous nocifensive behaviors. Here, we created a new apparatus, the thermal escape box, which can be attached to temperature-controlled plates and used to assess temperature-dependent effort-based decision-making. The apparatus consists of a light chamber with an opening that fits around temperature-controlled plates, and a small entryway into a dark chamber. A mouse must choose to stay in a brightly lit aversive area or traverse the plates to escape to the enclosed dark chamber. We quantified escape latencies of adult C57Bl/6 mice at different plate temperatures from video recordings and found they were significantly longer at 5 °C, 18 °C, and 52 °C, compared to 30 °C, a mouse's preferred ambient temperature. Differences in escape latencies were abolished in male Trpm8$^{-/-}$ mice and in male Trpv1$^{-/-}$ animals. Finally, we show that chronic constriction injury procedures or oxaliplatin treatement significantly increased escape latencies at cold temperatures compared to controls, the later of which was prevented by the analgesic meloxicam. This demonstrates the utility of this assay in detecting cold pain. Collectively, our study has identified a new and effective tool that uses cost-benefit valuations to study thermosensation and thermal pain.

## 1. Introduction

The ability to detect temperature and distinguish between those that are innocuous versus noxious is referred to as thermosensation and is essential to survival. Thermosensing guides behavior by informing decisions about which environments and objects are safe to explore and interact with, and which are not. Thermal pain, which includes thermal allodynia and thermal hyperalgesia, is a hallmark of many pathological conditions, such as chemotherapy-induced peripheral neuropathy (Descoeur et al., 2011; Sittl et al., 2012) and lumbar radiculopathy (Defrin et al., 2014), and can negatively impact involvement in daily activities. Numerous genetic models using both invertebrates and vertebrate animals have been developed to understand the molecular underpinnings of thermosensation and thermal pain.

A variety of behavioral assays can be used in rodents to analyze how genetic manipulations or pharmacological treatments affect physiological thermosensing and thermal pain. For example, the tail flick and Hargreaves assays measure response latencies for the detection of a radiant noxious heat stimulus applied to the tail or plantar surface of the hind paw, respectively (D'Amour and Smith, 1941; Hargreaves et al., 1988). A thermal probe test that involves placing a 2 mm thermal probe to the hind paw can be used to measure the temperature at which paw withdrawal occurs for quantification of heat thresholds, heat allodynia or hypoalgesia (Deuis and Vetter, 2016). A similar assay, referred to as the cold plantar assay, uses a dry ice probe to measure paw withdrawal latencies (Brenner et al., 2012). These assays have been widely used and

* Corresponding author at: 1275 Med Science Drive, Tupper Hall 4135, Davis, CA 95616, 530.754.2780, United States.
*E-mail addresses:* jrdayton@ucdavis.edu (J.R. Dayton), josmarquez@ucdavis.edu (J. Marquez), akromo@ucdavis.edu (A.K. Romo), ijchen@ucdavis.edu (Y.-J. Chen), jecontrer@ucdavis.edu (J.E. Contreras), tgriffith@ucdavis.edu (T.N. Griffith).
[1] These authors contributed equally to this publication.

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validated by many research groups. One caveat, however, is that the behavioral output that is quantified is a withdrawal response, a subjective measurement recorded by the researcher. It is also considered a spinal reflex that does not require the formation of a thermal or painful percept by higher brain centers (Irwin et al., 1951). The hot or cold plate assays (Allchorne et al., 2005; Woolfe and Macdonald, 1944), in which unrestrained rodents are placed on a metal plate set to a constant noxious temperature, are believed to engage supraspinal pathways (Giglio et al., 2006), but also require subjective analysis of nocifensive behaviors by an observer. Temperature preference assays, either the two-temperature choice test or the thermal gradient (Moqrich et al., 2005), can be used to analyze thermal aversion in freely moving rodents and objective measurements can be made by video acquisition and analysis software.

Behavioral choices are intrinsically linked to sensory input (Houweling and Brecht, 2008) and motivation to engage in certain behaviors is often impacted by pain states in which sensory signaling is distorted (Wiech and Tracey, 2013). There are a few behavioral assays available to assess the interaction between thermosensation or thermal pain and behavioral motivation, however, they require several days of training and that the animal learn the behavior that is being quantified (Baliki et al., 2005; Mauderli et al., 2000; Neubert et al., 2005; Reker et al., 2020). Here, we present the thermal escape box, which relies on the innate photophobia of mice to analyze temperature-dependent cost-benefit decision making through quantification of escape latencies. The apparatus consists of a light chamber that contains a small acrylic platform followed by an opening for the placement of temperature-controlled plates. Connected to the light chamber is an enclosed dark chamber that is accessible via a small entryway. In the thermal escape assay, the intrinsic motivation of the rodent to escape from the aversive environment in the light chamber to the dark chamber conflicts with the need to traverse metal plates set to a noxious temperature.

In the present study, we used adult C57Bl/6 mice of both sexes to validate the appropriate experimental design for use of the thermal escape box. Escape latencies were significantly longer for cool temperatures (18 °C), as well as temperatures in the noxious cold and heat range (5 °C and 52 °C, respectively). We determined that beginning testing with a noxious temperature and habituation to the apparatus can decrease escape latencies. We confirmed the utility of this assay for the study of physiological temperature sensing using mice lacking the cold-sensitive ion channel, TRPM8, and the heat-sensitive ion channel, TRPV1. We also show that compared to sham controls, escape latencies at noxious cold temperatures were significantly higher in mice who underwent chronic constriction injury (CCI) procedures. Furthermore, we show that treatment with the chemotherapeutic agent oxaliplatin, which is known to produce cold pain, resulted in significantly longer escape latencies at noxious cold temperatures, which was prevented by the analgesic meloxicam. Thus, this assay can also be used to assess the interaction between pain states and motivation. Collectively, this work provides evidence that supports the use of the thermal escape box in studies of mammalian thermosensation and preclinical pain research.

## 2. Materials and methods

### 2.1. Animals

C57Bl/6 (stock no. 000664), TRPV1$^{-/-}$ (stock no.003770) and TRPM8$^{-/-}$ (stock no. 008198) were obtained from The Jackson Laboratory. Mice were maintained on a 12 h light/dark cycle (7am/7pm) and fed *ad libitum* with irradiated 5058-PicoLab Mouse Diet 20 lab block. Humidity in the vivarium ranges from 30 % to 40 % with ambient temp between 68°F and 75°F. Water was also provided *ad libitum*. Genotyping was outsourced to Transnetyx. Mice were maintained in pathogen free conditions and the study's animal use was conducted according to guidelines from the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of UC Davis (#22438).

### 2.2. Testing environment

All behavioral testing was conducted in a quiet, isolated room maintained at ambient temperature and humidity. No other behavioral assays were conducted during testing sessions. Mice underwent room acclimation for 60 min prior to the start of behavioral testing on each experiment day. Mice were handled and transported to the testing room on 4–5 separate days before the start of data collection to habituate them to human contact and cart transport, respectively. The thermal escape box was always positioned in the exact same location of the testing room during test sessions to maintain consistent ambient light conditions. Individuals conducting behavioral testing did not wear any perfumes or heavily scented aromatics.

### 2.3. Apparatus

The thermal escape box consists of conjoined light (465 mm L x 185 mm W x 345 mm H) and dark (100 mm L x 185 mm W x 345 mm H) chambers (Fig. 1). The light and dark chambers are constructed from acrylic resin (50 mm thick) and are solid white and black, respectively. The thermal escape box was built by the UC Davis Translating Engineering Advances in Medicine (TEAM) Laboratory. The light chamber is open air with no lid and the dark chamber has a black lid and a narrowed entryway (40 mm x 40 mm) for access from the light chamber. In this study, the apparatus was placed on top of two metal temperature-controlled plates (Bioseb, BIO-T2CT) whose temperature is controlled by external software (T2CT v2). The metal plates fit into an opening in the bottom of the light chamber. When assembled, the plates were flush with the bottom of the light and dark chambers. During experimentation, the two plates were always set to the same temperature. A standard video camera is placed above the apparatus to record each trial, keeping investigator interference to a minimum.

### 2.4. Assay

8–12-week-old male and female mice were placed individually in the center of the acrylic platform of the light chamber. Latency to escape to the dark chamber was video recorded and analyzed *post hoc*. Trial timing began when all four paws touched down on the white acrylic platform. Trial time ended when all four paws entered the dark chamber or after 180 s, which ever came first. Escape latency was recorded at each temperature, with one trial per temperature for each mouse. After each trial, the mouse is returned to their home cage and allowed to reacclimate for at least 15 min before beginning the next trial. The apparatus and metal plates were cleaned with a 10 % bleach solution in between trials. Except for data shown in Fig. 6, all trials were performed on the same day.

### 2.5. Chronic constriction injury procedures

A chronic constriction injury (CCI) was produced by ligation of the left common sciatic nerve in male and female 10–12-week-old C57Bl/6 mice. Following standard aseptic techniques for survival surgery in rodents, mice were anesthetized in an induction chamber using 5 % isoflurane in $O_2$, then anesthesia was maintained during surgery via nose cone delivering isoflurane at 1–5 % in $O_2$. Lubricating ophthalmic ointment was applied to the animal's eyes to prevent drying and post-surgical discomfort. The animal was placed onto a thermo-regulated heating mat at 37 °C. The left hind leg of the animal was shaved and sterilized with three alternating applications of 70 % isopropyl alcohol and iodine solution. An incision in the skin was made 3–4 mm below the femur and a cut was made through the connective tissue between the gluteus superficialis and the biceps femoris muscles. A retractor was used to widen the gap between the two muscles, allowing clear

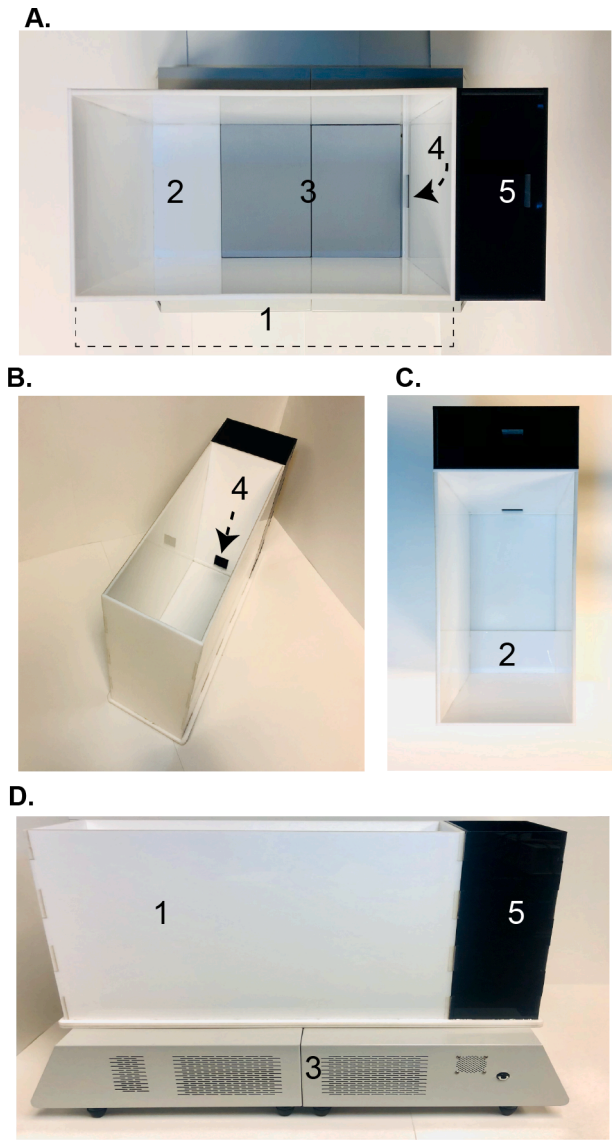


**Fig. 1. Thermal Escape Box Apparatus. (A)** An arial view of the thermal escape box showing the light chamber containing an opening for insertion of temperature-controlled plates, and the entry way into the dark chamber. **(B-C)** Arial views of the thermal Escape Box apparatus, and **(D)** a side view of the apparatus mounted on two temperature-controlled plates, with components labeled **1**: Open-air light chamber, **2**: Acrylic platform, **3**: Temperature controlled plates, **4**: Entry into dark chamber, **5**: Closed dark chamber.

visualization of the sciatic nerve. Approximately 10 mm of the sciatic nerve (proximal to the sciatic trifurcation) was freed from the surrounding connective tissue. Four ligatures (chromic gut 4.0) were tied with a double knot, 1 mm apart, proximal to the trifurcation of the sciatic nerve. A second loop was placed on top of the first to complete the knot. The loose ends of the ligature were cut to around 1 mm. Constriction of the nerve in this manner is minimal and immediately stopped if a brief twitch is observed to prevent arresting of the epineural blood flow. Chromic gut sutures were used to close the muscle layer, and non-absorbable sutures (prolene 5.0) were used to close the skin. Mice were assayed in the thermal escape box 12 days post operation to allow for post-surgical inflammation to subside and for mice to reacclimate to ambient cage conditions following injury. Sham animals underwent the same surgical procedures but in the absence of ligature placement.

### 2.6. Chemotherapeutic induced cold allodynia

Cold allodynia was induced using the chemotherapeutic drug oxaliplatin (Sigma Aldrich, CAS #: 61825–94-3). The compound was dissolved in a sterile vehicle of 5 % glucose at a concentration of 1 mg/ml. 8–10-week-old C57Bl/6 female mice were injected intraperitoneally for five consecutive days with oxaliplatin (3 mg/kg body weight) or vehicle (5 % glucose), followed by five consecutive days of rest, and a final five-day course of oxaliplatin for a cumulative dose of 30 mg/kg. Mice were assayed five days after the final injection, and twenty-one days after the initial injection. For treatment with analgesia, 8–10-week-old C57Bl/6 female mice were treated with oxaliplatin as described, however, four hours prior to testing, mice were given a subcutaneous injection of meloxicam (5 mg/kg, NDC:13985–559-10) or vehicle (0.9 % bacteriostatic saline). Meloxicam was diluted in sterile 0.9 % bacteriostatic saline to a concentration of 0.5 mg/ml prior to injection.

### 2.7. Statistical analysis

Summary data are presented as mean ± SEM, from n animals. Data normality was determined by the Shapiro-Wilk test. Statistical differences were determined using a repeated measures nonparametric Friedman's One-Way ANOVA or a repeated measures Two-Way ANOVA. For One-Way ANOVAs that reached significance, a Dunn's *post hoc* test was used. For Two-Way ANOVAs that reached significance, a Tukey's multiple comparisons *post hoc* test was used. Statistical tests are listed in the main text and/or figure legends. Statistical significance in each case is denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Statistical tests were performed using Prism 10.0 (GraphPad Software). All data generated or analyzed during this study are included in the article.

## 3. Results

### 3.1. Analysis of physiological thermosensing

We assayed 8–10-week-old male and female C57Bl/6 mice in the thermal escape box and quantified escape latencies for four different temperatures. Temperatures were presented in the following order: 30 °C, 5 °C, 18 °C, and 52 °C. Mean escape latencies for males were: 5 °C: 112.3 s ± 13.96 s; 18 °C: 98.43 s ± 14.02 s; 30 °C: 43.33 s ± 6.216 s; 52 °C: 129.0 s ± 15.73 s. Mean escape latencies for females were: 5 °C: 73.52 s ± 13.75 s; 18 °C: 79.68 s ± 12.56 s; 30 °C: 27.72 s ± 4.363 s; 52 °C: 98.2 s ± 13.65 s. For males, statistical analyses found that escape latencies were significantly longer for 5 °C and 52 °C as compared to 30 °C (Fig. 2A, Video 1). For females, compared to 30 °C, all other temperatures tested showed significantly longer escape latencies (Fig. 2B). This demonstrates the thermal escape box serves as a cost-benefit valuation assay that can be used to examine physiological thermosensing.

### 3.2. Assay design considerations

We next asked whether beginning the assay with a noxious temperature would affect escape latencies in subsequent trials. We assayed mice using the following temperature order: 5 °C, 18 °C, 30 °C and 52 °C. Mean escape latencies for males were: 5 °C: 112.6 s ± 16.2 s; 18 °C: 77.82 s ± 15.44 s; 30 °C: 86.18 s ± 15.66 s; 52 °C: 136.9 s ± 15.51 s. Mean escape latencies for females were: 5 °C: 124.3 s ± 14.93 s; 18 °C: 89.2 s ± 14.05 s; 30 °C: 77.85 s ± 13.76 s; 52 °C: 134.5 s ± 15.9 s. Male and female escape latencies across temperatures were not significantly different, except when comparing escape latencies at 30 °C and 52 °C. Thus, beginning the assay with a trial at 5 °C led to longer escape latencies in subsequent trials (Fig. 3).

We also found that habitation to the thermal escape box apparatus for 30 min the day before behavioral testing eliminated significant

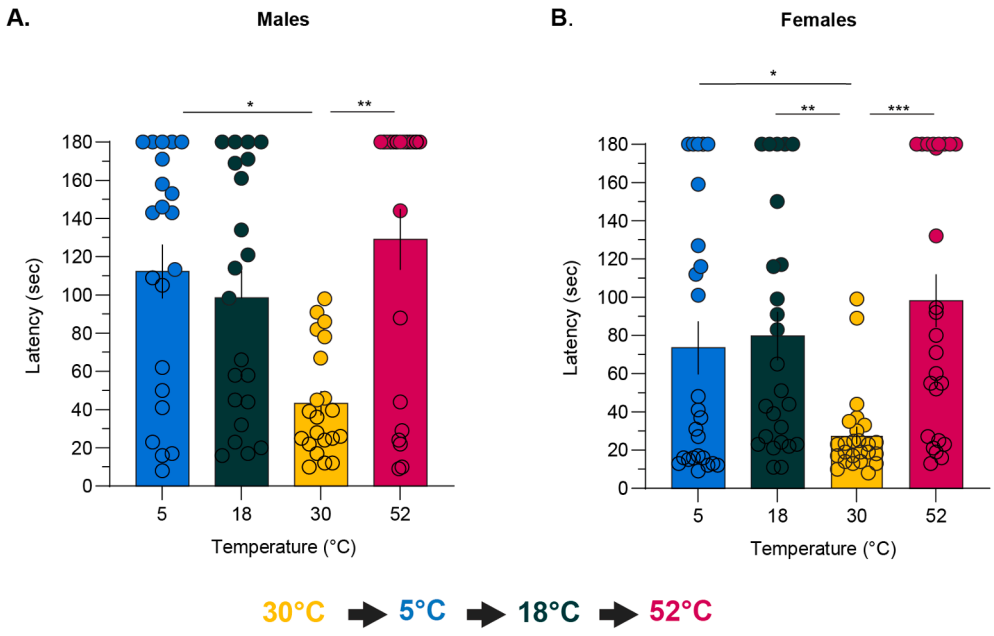


**Fig. 2. The Thermal Escape Box can be used to measure physiological thermosensing.** 8–10 week old C57Bl/6 male (**A**, n = 21 mice; 5 °C vs. 30 °C, p = 0.0305, and 30 °C vs 52 °C, p = 0.0016) and female (**B**, n = 25 mice; 5 °C vs. 30 °C, p = 0.0158; 18 °C vs. 30 °C, p = 0.004; and 30 °C vs. 52 °C, p = 0.0004) mice were assayed in the thermal escape box assay at 4 different temperatures. The temperature order was as follows: 30 °C, 5 °C, 18 °C, and 52 °C. Significance was determined using a Friedman's One-way ANOVA (**A**-P = 0.0030; **B**-P = 0.0010) with Dunn's multiple comparisons: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Individual dots represent biological replicates. **Video 1.** A C57Bl/6 mouse readily transverses plates set to a preferred temperature of 30 °C, but takes considerably longer to transverse plates set to 5 °C.

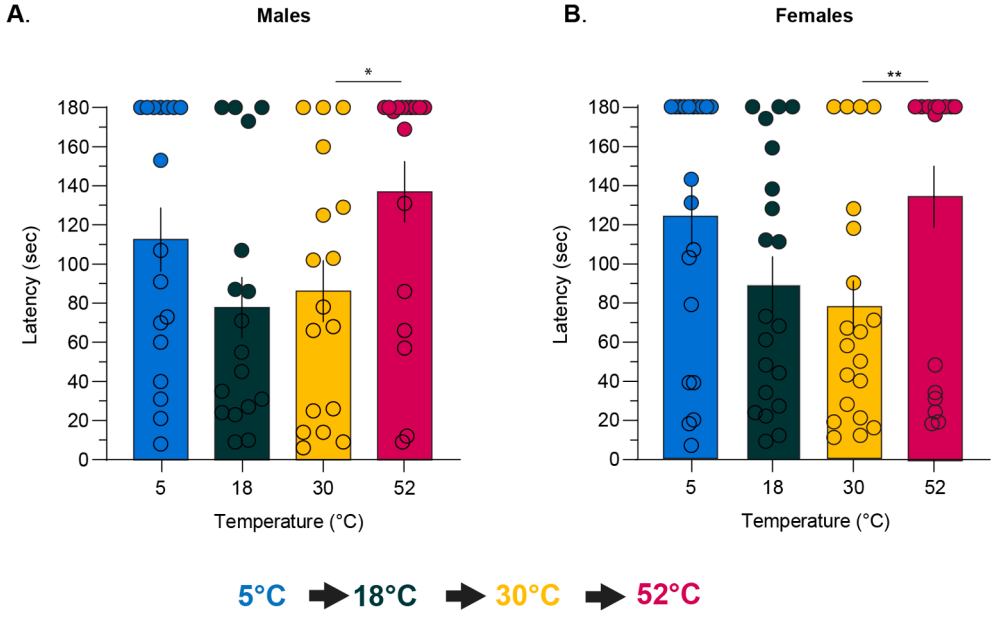


**Fig. 3. Starting the Thermal Escape Box Assay with a noxious temperature increases escape latencies at innocuous temperatures.** 8–10-week-old C57Bl/6 mice of both sexes were assayed in the thermal escape box at 4 different temperatures. The temperature order was as follows: 5 °C, 18 °C, 30 °C and 52 °C. Significant differences were observed between 30 °C and 52 °C in males (**A**, n = 17 mice, p = 0.0171) and females (**B**, n = 20 mice; p = 0.0044). Significance was determined using a Friedman's One-way ANOVA (**A**-P = 0.0164; **B**-P = 0.0363) with Dunn's multiple comparisons. Individual dots represent biological replicates.

differences in escape latencies (Fig. 4, temperature order: 30 °C, 5 °C, 18 °C, and 52 °C). The average escape latencies for each temperature tested were 5 °C: 81.56 s ± 30.99 s; 18 °C: 17.22 s ± 9.973 s; 30 °C: 5.778 s ± 1.331 s, 52 °C: 22.56 s ± 13.34 s. We did not observe differences in escape latencies when comparing any temperature to 30 °C. Thus, in addition to starting the assay with a noxious temperature, habituation to the thermal escape box apparatus can affect escape latencies.

We next examined the effect of repeat testing on the same day, or repeat testing during a one-week period, on escape latencies. For same day testing, mice were assayed three times on the same day, with 90 min in between each trial. To limit total testing time, two temperatures were chosen, 30 °C and 5 °C. The average escape latencies for each temperature were: Trial 1: 30 °C: 53.60 s ± 15.11 s, 5 °C: 97.30 s ± 20.30 s;

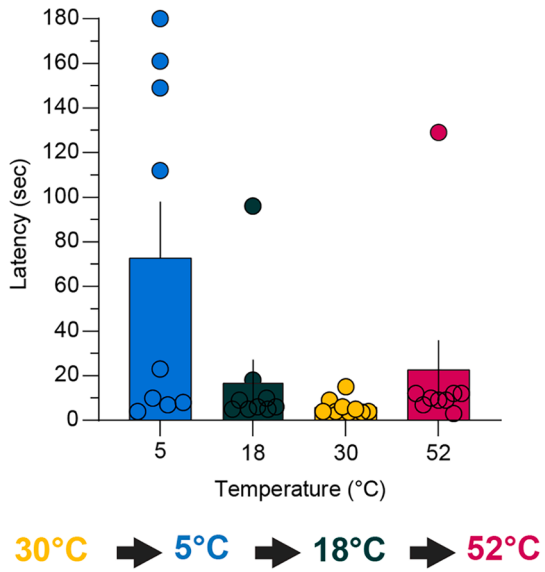


**Fig. 4. Habituation to the thermal escape box apparatus impairs performance.** Mice were habituated to the apparatus for 30 min one day prior to behavioral testing. No significant differences in escape latencies were observed between temperatures. Male and female mice were combined (n = 9). Significance was determined using a Friedman's One-way ANOVA (P = 0.0364) with Dunn's multiple comparisons. Individual dots represent biological replicates.

Trial 2: 30 °C: 86.10 s ± 22.72 s, 5 °C: 168.3 s ± 7.817 s; Trial 3: 30 °C: 147.0 s ± 18.71 s, 5 °C: 160.4 s ± 17.49 s. Within each temperature, escape latencies significantly increased with subsequent trials (Fig. 5A). Additionally, statistical significance was lost by the third trial when comparing escape latencies at 30 °C to 5 °C (Fig. 5 B). For repeat testing during a one-week period, mice were assayed three times, and each trial was separated by one day. The following temperature order was used during each trial: 30 °C, 5 °C, 18 °C, and 52 °C. Escape latencies for 30 °C and 52 °C remained stable over the three trials; however, there were significant increases in escape latencies at 5 °C and 18 °C following repeat testing during the same week (Fig. 6, mean ± SEM values can be found in the figure legend). Collectively, these data indicate that repeat testing on the same day or on different days can be carried out, though the potential for increased escape latencies should be considered during experimental planification.

### 3.3. Analysis of thermosensing deficits

The thermotransduction channels TRPM8 and TRPV1 are activated by cold and heat, respectively (Caterina et al., 1999; McKemy et al., 2002). To determine if the thermal escape box can be used to detect deficits in thermosensation, we assayed male and female TRPM8$^{-/-}$ and TRPV1$^{-/-}$ mice, which have known thermosensory deficits (Bautista et al., 2007; Caterina et al., 2000). We first tested TRPM8$^{-/-}$ males and female mice at 30 °C, followed by trials at 5 °C, 18 °C and 52 °C (Fig. 7 A, B). Mean escape latencies for TRPM8$^{-/-}$ males were: 5 °C: 29.15 s ± 10.12 s; 18 °C: 34.33 s ± 8.281 s; 30 °C: 33.62 s ± 4.464 s; 52 °C: 63.77 s ± 14.82 s. Mean escape latencies for TRPM8$^{-/-}$ females were: 5 °C: 26.5 s ± 8.9 s; 18 °C: 13.43 s ± 1.68 s; 30 °C: 25.43 s ± 3.587 s; 52 °C: 28.5 s ± 8.338 s. For male mice, statistical analyses found no significant differences in escape latencies compared to 30 °C. For female mice, escape latencies at 18 °C were slightly but significantly faster compared to 30 °C, though this is consistent with TRPM8 being important for detecting cool temperatures. We next assayed male and female TRPV1$^{-/-}$ mice at 30 °C, followed by trials at 5 °C and 52 °C (Fig. 7 C, D). We did not anticipate any effect of loss of TRPV1 on temperature-sensing at 18 °C; thus, we excluded this temperature when testing TRPV1$^{-/-}$ mice. Mean escape latencies for TRPV1$^{-/-}$ males were: 5 °C: 53.83 s ± 16.0 s; 30 °C: 31.83 s ± 5.352 s; 52 °C: 47.0 s ± 13.37 s. Mean escape latencies for TRPV1$^{-/-}$ females were: 5 °C: 79.18 s ± 14.42 s; 30 °C: 24.09 s ± 2.632 s, 52 °C: 78.23 a ± 12.85 s. We observed no significant differences in mean escape latencies for male Trpv1$^{-/-}$ mice compared to either 5 °C or 52 °C. Conversely, female TRPV1$^{-/-}$ mice did have significantly slower escape latencies at 5 °C and 52 °C compared to 30 °C. Nevertheless, these data highlight the utility of the thermal escape box for the detection of deficits in thermosensation.

### 3.4. Analysis of thermal hyperalgesia

Finally, we chose the two different neuropathic pain paradigms to determine if the thermal escape box can be used to analyze thermal pain. The first paradigm we tested in the thermal escape box was chronic

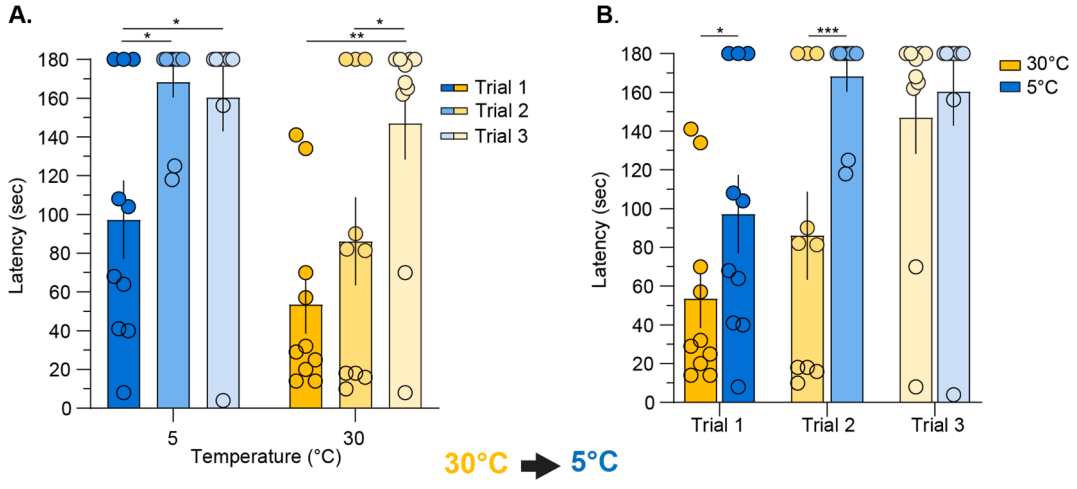


**Fig. 5. Same day repeat testing in the thermal escape box lengthens escape latencies.** 8–10-week-old C57Bl/6 mice of both sexes were assayed in the thermal escape box assay at two different temperatures three times in the same day, with 90 min between trials. The temperature order was as follows: 30 °C, 5 °C. (**A**) Significant differences were observed between the trials at 5 °C (n = 10 mice, Trial 1 vs. Trial 2, p = 0.0171; Trial 1 vs. Trial 3, p = 0.0380) and at 30 °C (n = 10 mice, Trial 1 vs. Trial 3, p = 0.0013; Trial 2 vs. Trial 3, p = 0.03469). (**B**) Significant differences were observed between the temperatures in the first trial (n = 10 mice, 5 °C vs. 30 °C, p = 0.0442) and the second trial (n = 10 mice, 5 °C vs. 30 °C, p = 0.0005). No significant differences in escape latencies were observed in the third trial. Significance was determined using a Two-way ANOVA (Row Factor x Column Factor: F (2, 27) = 2.774, P = 0.0802; Row Factor: F (1, 27) = 15.09, P = 0.0006; Column Factor: F (2, 27) = 7.724, P = 0.0022) with Tukey's multiple comparisons. Individual dots represent biological replicates.

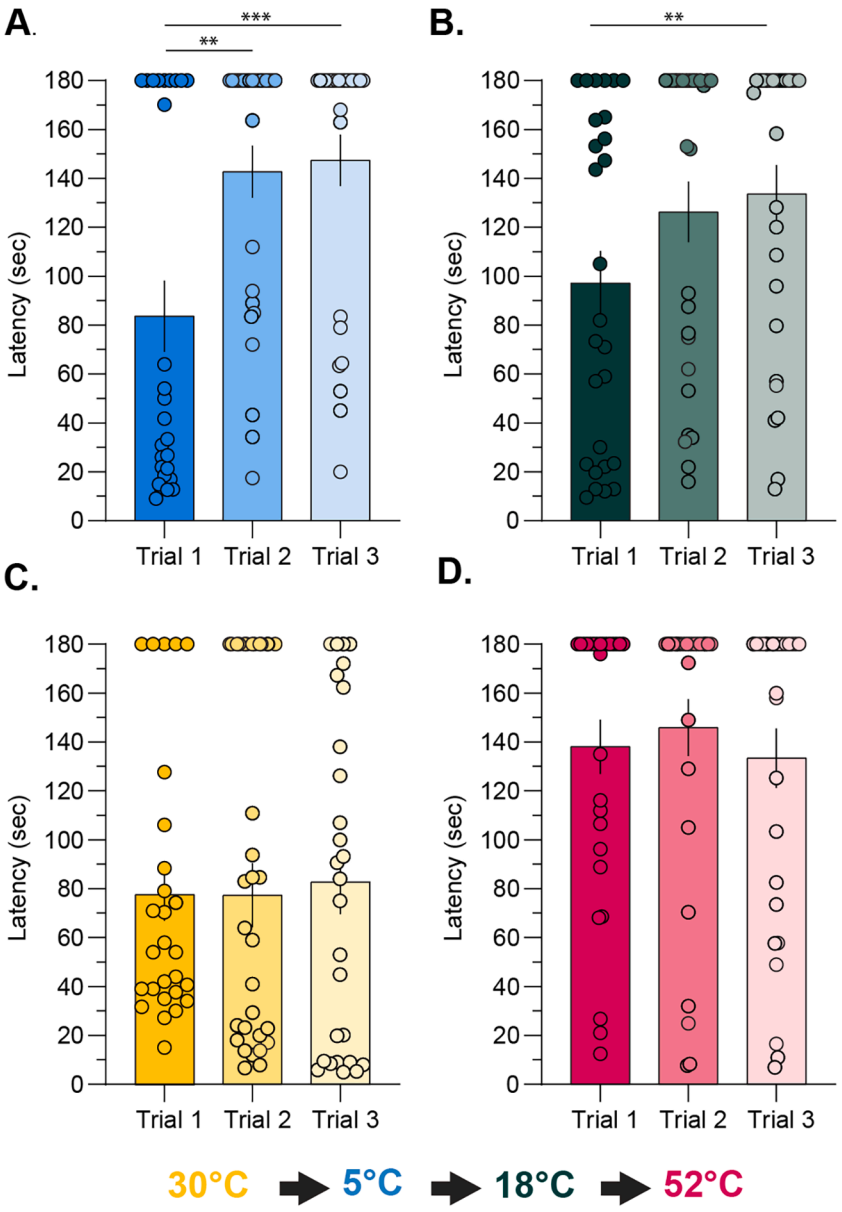


**Fig. 6. Repeat testing with the thermal escape box within the same week can impact performance.** 8–10-week-old C57Bl/6 mice of both sexes were assayed in the thermal escape box assay at four different temperatures three times in the same week, with subsequent runs on every other day. The temperature order was as follows: 30 °C, 5 °C, 18 °C, and 52 °C. Significant differences were observed at 5 °C between Trial 1 and Trial 2 (**A**, n = 27 mice, p = 0.0016) and Trial 1 and Trial 3 (**A**, n = 27 mice, p = 0.0003), F-value = 14.15. Significant differences were observed at 18 °C between Trial 1 and Trial 3 (**B**, n = 27 mice, p = 0.0062). No significant differences in escape latencies were observed at 30 °C and 52 °C (**C, D**). Significance was determined using a Friedman's One-way ANOVA (**A**-P = 0.0001; **B**-P = 0.0001; **C**-P = 0.3914; **D**-P = 0.3311) with Dunn's multiple comparisons. Individual dots represent biological replicates. The average escape latencies for each temperature were: Trial 1—5 °C: 83.64 s ± 14.56 s, 18 °C: 97.14 s ± 13.2 s; 30 °C: 77.74 s ± 10.70 s, 52 °C: 138.1 s ± 11.05 s; Trial 2—5 °C: 142.8 s ± 10.64 s, 18 °C: 126.3 s ± 12.37 s; 30 °C: 77.37 s ± 13.12 s, 52 °C: 145.9 s ± 11.63 s; Trial 3—5 °C: 147.4 s ± 10.55 s, 18 °C: 133.7 s ± 11.62 s; 30 °C: 82.75 s ± 13.12 s, 52 °C: 133.4 s ± 12.18 s.

constriction injury (CCI). CCI mimics peripheral nerve injury and is one of the most widely used models to study neuropathic pain (Austin et al., 2012). One of the most common pain behaviors observed following CCI is thermal hyperalgesia (Sheehan et al., 2021). Male and female 10–12-week-old C57Bl/6 mice underwent CCI or sham procedures. 12-days post injury, animals were assayed in the thermal escape box. We excluded testing at 5 °C and 52 °C from this analysis as we predicted escape latencies for sham mice at these temperatures would already be high (Fig. 2), making differences difficult to assess. Mice were first tested mice at the innocuous temperature of 30 °C, followed by trials at 18 °C and 10 °C. Mean escape latencies were for male and female mice combined were 10 °C: 73.07 s ± 10.29 s; 18 °C: 20.90 s ± 2.541 s; 30 °C: 19.33 s ± 1.304 s for CCI animals. Mean escape latencies for sham mice were 10 °C: 26.38 s ± 6.138 s; 18 °C: 16.13 s ± 2.573 s; 30 °C: 19.63 mean ± 4.855 s (Fig. 8). Statistical analyses found escape latencies were significantly longer for CCI mice at 10 °C compared to sham mice, demonstrating the thermal escape box can be used to quantify injury-induced thermal hyperalgesia.

We next examined the utility of the thermal escape box in detecting cold pain using the oxaliplatin-induced cold allodynia model. Female mice were injected intraperitoneally for five consecutive days with oxaliplatin (3 mg/kg) or vehicle (5 % glucose), followed by five consecutive days of rest and a final five-day course of oxaliplatin, for a cumulative dose of 30 mg/kg (Braden et al., 2022). Mice were assayed

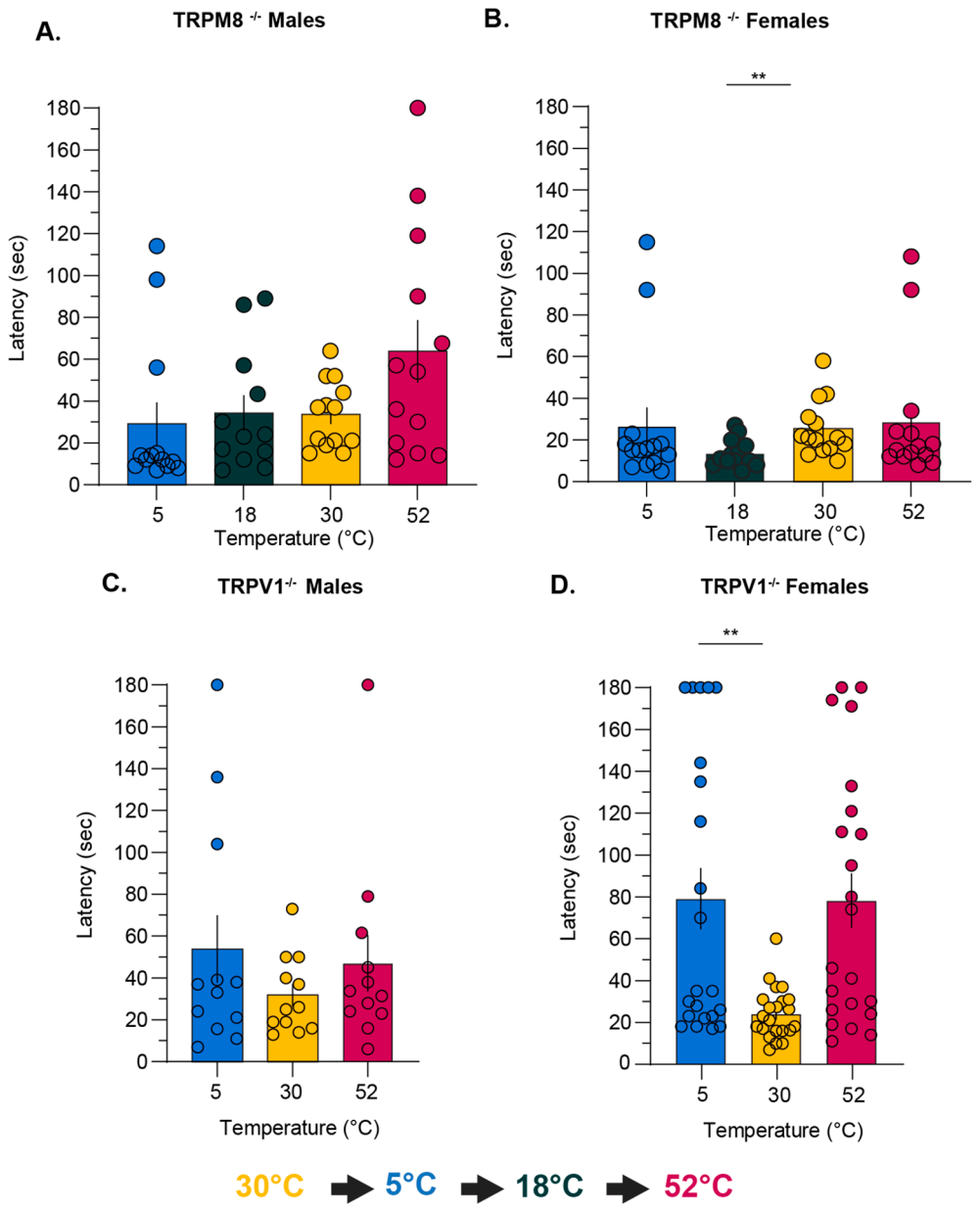


**Fig. 7. Validation of the thermal escape box using mice with established thermosensory deficits.** 8–10-week-old TRPM8$^{-/-}$ and TRPV1$^{-/-}$ mice of both sexes were assayed in the thermal escape box assay. The temperature order for TRPM8$^{-/-}$ mice was as follows: 30 °C, 5 °C, 18 °C, and 52 °C. The temperature order for TRPV1$^{-/-}$ mice was as follows: 30 °C, 5 °C, and 52 °C. No significant differences in escape latencies were observed in TRPM8$^{-/-}$ males (**A**, n = 13 mice). Significant differences were observed in TRPM8$^{-/-}$ females (**B**, n = 14 mice, 18 °C vs. 30 °C, p = 0.0030). No significant differences in escape latencies were observed in Trpv1$^{-/-}$ males (**C**, n = 12 mice). Significant differences were observed in TRPV1$^{-/-}$ females (**D**, n = 22 mice, 5 °C vs. 30 °C, p = 0.0019). Significance was determined using a Friedman's One-way ANOVA (**A**-P = 0.0746; **B**-P = 0.0073; **C**-P = 0.5097; **D**-P = 0.0017) with Dunn's multiple comparisons. Individual dots represent biological replicates.

five days after the final dose of oxaliplatin and escape latencies were quantified for the following temperature order: 30 °C, 18 °C and 5 °C. Mean escape latencies were: 5 °C: 80.38 s ± 13.79 s; 18 °C: 19.70 s ± 1.856 s; 30 °C: 24.36 s ± 2.929 s for oxaliplatin-treated mice. Mean escape latencies for vehicle-injected mice were 5 °C: 44.21 s ± 16.54 s; 18 °C: 18.67 s ± 1.258 s; 30 °C: 12.48 mean ± 1.593 s. Statistical analyses show that escape latencies were significantly longer for oxaliplatin-injected mice at 5 °C compared to vehicle-injected controls (Fig. 9). Collectively, these results demonstrate the thermal escape box can be used to quantify chemotherapy-induced cold pain.

Finally, we asked if quantification of escape latencies in the thermal escape box were sensitive to analgesic drug affects. To accomplish this, we treated female mice with oxaliplatin as described above, however, 4 h prior to testing, half of the cohort was given a subcutaneous injection of the analgesic meloxicam (5 mg/kg), whereas the other half received vehicle (saline). Mice were assayed using the following temperature order: 30 °C, 18 °C and 5 °C. Mean escape latencies for meloxicam-treated mice were 5 °C: 60.37 s ± 26.12 s; 18 °C: 26.58 s ± 7.175 s; 30 °C: 26.50 s ± 6.674 s. Mean escape latencies for vehicle-injected mice were: 5 °C: 109.1 s ± 19.64 s; 18 °C: 26.71 s ± 7.091 s; 30 °C: 24.17 mean ± 2.656 s. Meloxicam treatment significantly reduced escape latencies at 5 °C compared to vehicle treatment (Fig. 10), showing that analgesic efficacy can be determined using the thermal escape box assay.

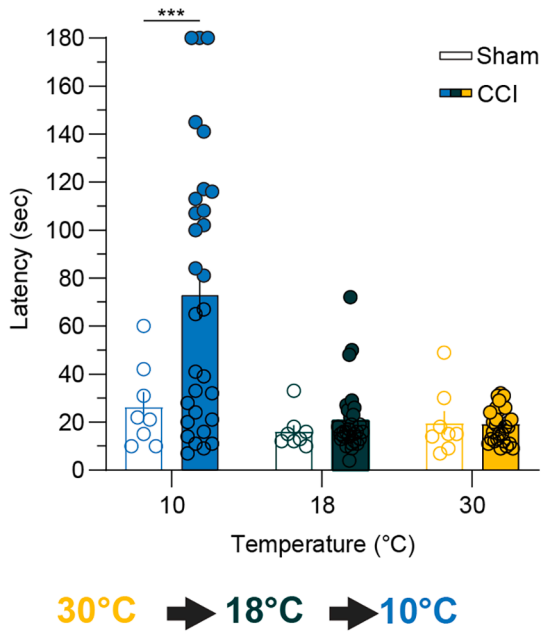


**Fig. 8. The thermal escape box detects the effect of thermal pain on effort-based decision making in the chronic constriction injury model.** 10–12-week-old C57Bl/6 mice of both sexes were assayed in the thermal escape box assay at 12 days post CCI procedures. The temperature order was as follows: 30 °C, 18 °C, and 10 °C. Significant differences in escape latencies were observed between CCI and sham groups at 10 °C (CCI n = 30 mice and sham n = 8 mice, p = 0.0004). Significance was determined using a Two-way ANOVA (Row Factor x Column Factor: F (2, 72) = 4.817, P = 0.0109; Row Factor: F (1.119, 40.27) = 9.123, P = 0.0033; Column Factor: F (1, 36) = 4.943, P = 0.0326) with Tukey's multiple comparisons. Filled bars represent CCI-treated mice and clear bars represent sham controls. Individual dots represent biological replicates.

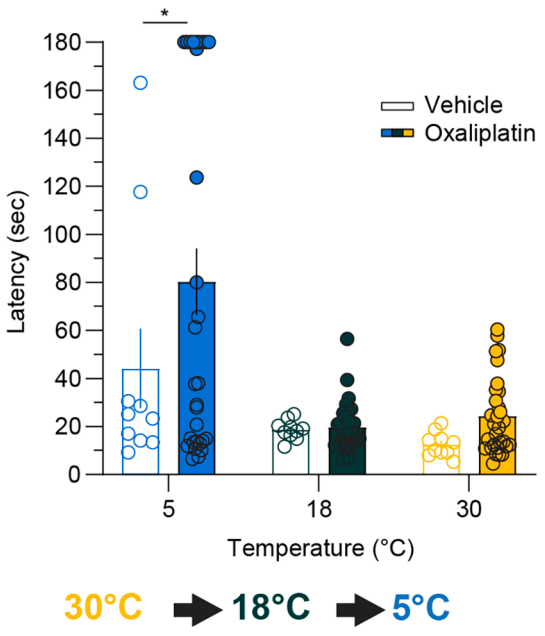


**Fig. 9. The thermal escape box detects the effect of thermal pain on effort-based decision making in chemotherapeutic induced cold allodynia.** 10–12-week-old C57Bl/6 female mice were assayed in the thermal escape box assay following induced cold allodynia from chronic oxaliplatin injection. Mice were assayed at 5 days post final injection. The temperature order was as follows: 30 °C, 18 °C, and 5 °C. Significant differences in escape latencies were observed between oxaliplatin and vehicle groups at 5 °C (oxaliplatin n = 30 mice and vehicle n = 10 mice, p = 0.02). Significance was determined using a Two-way ANOVA (Row Factor x Column Factor: F (2, 76) = 1.479, P = 0.2343; Row Factor: F (1.085, 42.23) = 11.53, P = 0.0012; Column Factor: F (1, 38) = 3.008, P = 0.0910) with Tukey's multiple comparisons. Filled bars represent oxaliplatin-treated mice and clear bars represent vehicle controls. Individual dots represent biological replicates.

## 4. Discussion

In the current study, we present results that support use of the thermal escape box to study mechanisms of physiological thermosensation and thermal pain. This assay relies on unlearned, naturalistic escape behaviors to evaluate how temperature affects cost-benefit decision making. The thermal escape box apparatus forces mice to choose between staying in an aversive, brightly lit chamber, or traversing temperature-controlled metal plates to escape to a covered dark chamber. We found that wild-type mice readily crossed plates set to 30 °C, a mouse's preferred ambient temperature. Conversely, deviation from this preferred temperature resulted in significantly longer escape latencies, suggesting mice took more time to evaluate the cost-benefit relationship of experiencing non-preferred temperatures to avoid an aversive environment. Performance in this assay does not require training, and we validated the utility of this assay for detecting deficits in thermosensation using Trpm8$^{-/-}$ and Trpv1$^{-/-}$ mice. Finally, we found the thermal escape box can detect the presence of thermal pain in two different preclinical neuropathic pain models and can also be used to determine the efficacy of analgesic drugs. Thus, the thermal escape box is a novel decision-based behavioral paradigm for the study of thermosensation and thermal pain.

### 4.1. Cognitive aspects of thermosensation and thermal pain

Several behavioral assays exist and are widely used to test rodent responses to thermal stimuli, including the tail flick and Hargreaves assays, the hot/cold plate, the cold plantar assay, the two-temperature preference test, and the thermal gradient (Allchorne et al., 2005; Brenner et al., 2012; D'Amour and Smith, 1941; Deuis and Vetter, 2016; Hargreaves et al., 1988; Moqrich et al., 2005; Woolfe and Macdonald, 1944). Most of these assays rely on reflexive withdrawal responses, which involve spinal reflex circuitry but not supraspinal circuits (Irwin et al., 1951). Temperature sensing is initiated by the activation of sensory neurons in the peripheral nervous system, making reflexive assays quite useful for determining how genetic manipulations impair the function of these neurons. Nevertheless, the formation of a thermal percept and its associated salience and valence are constructed in the brain (Vestergaard et al., 2023), and behavioral assays that engage supraspinal regions are needed to understand how thermal stimuli guide behavior. Furthermore, pain is now recognized as a complex biological phenomenon that not only involves detection of noxious stimuli, but is also heavily influenced by social, emotional, and affective variables (Raja et al., 2020). While reflexive assays are adept for the analysis of nociceptive behaviors, operant and decision-based assays provide insight into the cognitive component of pain states, which has important translational relevance for preclinical pain research.

Indeed, the choice presented to mice in the thermal escape box assay is similar to decisions that people face when suffering from thermal dysesthesia. Thermal allodynia and hyperalgesia are often present in patients with neuropathic pain conditions and can affect various aspects of daily life. For example, patients treated with the chemotherapy drug oxaliplatin frequently develop cold allodynia and often choose to avoid cool beverages and environments with air conditioning, as both are prone to evoke painful sensations (Krishnan et al., 2005; Tofthagen, 2010; Tofthagen et al., 2013). While the latency to make these decisions has not been quantified, deciding to escape from the light chamber to the dark chamber based on plate temperature in the thermal escape box assay mimics the cost-benefit decisions people with thermal dysesthesia

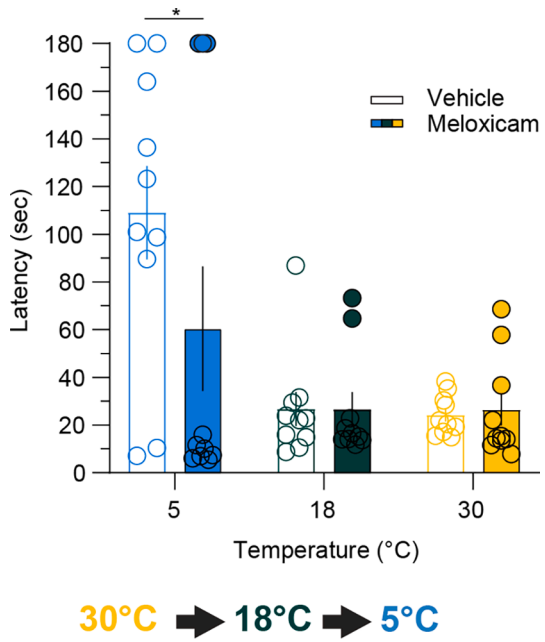


**Fig. 10. The thermal escape box detects the effect of thermal pain on effort-based decision making in chemotherapeutic induced cold allodynia.** 10–12-week-old C57Bl/6 female mice were assayed in the thermal escape box assay at following induced cold allodynia from chronic oxaliplatin injection. Mice were assayed at 5 days post final injection. 4 h prior to being assayed, mice were injected with the analgesic meloxicam (5 mg/kg), or a saline vehicle control. The temperature order was as follows: 30 °C, 18 °C, and 5 °C. Significant differences in escape latencies were observed between meloxicam and vehicle groups at 5 °C (meloxicam n = 10 mice and vehicle n = 10 mice, p = 0.0193). Significance was determined using a Two-way ANOVA (Row Factor x Column Factor: F (2, 36) = 2.280, P = 0.1169; Row Factor: F (2, 36) = 11.53, P < 0.0001; Column Factor: F (1, 18) = 1.455, P = 0.2434) with Tukey's multiple comparisons. Filled bars represent meloxicam-treated mice and clear bars represent vehicle controls. Individual dots represent biological replicates.

often make. This supports the quantification of escape latencies in this assay to assess the motivational and affective dimensions of thermosensory- and thermal pain-behaviors in genetic and preclinical models.

### 4.2. Decision-based assays to evaluate thermosensation and thermal pain

A variety of behavioral assays have been developed that take advantage of rodents' innate photophobia to test decision making, and similar effort-based decision-making somatosensory assays have been developed in the context of tactile sensitivity and mechanical pain. In the place escape/avoidance paradigm (Fuchs and McNabb, 2012), mice are presented with a noxious mechanical stimulus while in a dark chamber and provided the opportunity to escape to an aversive light chamber. The mechanical conflict system uses a similar premise as the thermal escape box and quantifies escape latencies from a light chamber to a dark chamber, but replaces temperature-controlled plates with height-adjustable nociceptive probes (Harte et al., 2016). An orofacial pain assessment device has also been developed, in which a mouse can acquire a food or water reward through an opening lined with metal wires, thus requiring contact with a noxious mechanical stimulus to obtain the reward. The metal wired-lined opening can also be replaced by a temperature-controlled thermode to adapt this assay for analysis of thermal pain (Nolan et al., 2011).

In addition to this operant orofacial thermal pain system, a few additional operant and decision-based thermosensory behavioral assays have been developed. The Algo Track (Baliki et al., 2005) and the Escapetest systems (Mauderli et al., 2000) measure escape latency from a metal plate set to noxious temperature to a plate set to ambient temperature. Both assays, however, require learning and several training sessions to optimize performance. The operant plantar thermal assay is a decision-based thermal test that also uses a conflict paradigm in which rodents must remain on a metal plate set to a noxious temperature to receive a water reward (Reker et al., 2020). This assay also requires several days of training to optimize behavioral performance and is dependent upon reward salience.

In contrast, the thermal escape box does not require training or a reward of sufficient strength to enhance behavioral performance. Indeed, habituation to the apparatus had a negative impact on behavior during the assay (Fig. 4). The thermal escape box instead leverages the innate motivation of a mouse to escape from the light chamber and places this in conflict with the need to cross temperature-controlled plates to do so.

### 4.3. Quantification of thermosensory deficits and thermal pain

Importantly, we used genetically modified mice with established temperature-sensing deficits to validate the efficacy of the thermal escape box (Fig. 7). TRPM8 is a cold-sensitive ion channel expressed in a subset of sensory neurons that comprise a labeled line for cold detection (Knowlton et al., 2013). Mice lacking this channel have deficits in cold detection, and in the thermal escape box assay, the escape latencies of TRPM8$^{-/-}$ mice were temperature-independent. This demonstrates a potential utility of the thermal escape box in detecting deficits in cold sensing. We also assayed TRPV1$^{-/-}$ mice. In male mice, performance in the thermal escape box was temperature independent. In female mice, however, temperature did have a significant effect on escape latencies. TRPV1 is activated by heat, though its role in temperature sensing is more complex than that of TRPM8 (Paricio-Montesinos et al., 2020; Woodbury et al., 2004). Triple knockout of TRPV1, TRPA1, and TRPM3 is required to completely abolish noxious heat sensation (Vandewauw et al., 2018). Furthermore, TRPV1 activity is regulated by sex hormones (Diogenes et al., 2006; Seol and Chung, 2022; Yang et al., 2023) which could explain the sex differences between TRPV1$^{-/-}$ mice in the thermal escape box. Indeed, sex-dependent differences in TRPV1 activity have been reported (Bubb et al., 2013; Huckleberry et al., 2023; Lafoux et al., 2020). Taken together, our results provide evidence that the thermal escape box can be used to detect changes in physiological temperature sensing.

The CCI model is a widely used preclinical neuropathic pain paradigm and gives rise to thermal allodynia. Our analysis of CCI and sham mice escape latencies in the thermal escape box found that CCI animals had longer escape latencies at 10 °C compared to sham controls. Interestingly, we did not observe significant differences in escape latencies for CCI animals at 18 °C, though naïve animals had significantly longer escape latencies at 18 °C compared to 30 °C. It is possible that the thermal hypersensitivity induced by CCI injury motivates escape behavior up to a certain temperature threshold. Similar findings were reported for the mechanical conflict system (Harte et al., 2016).

We also tested mice injected with oxaliplatin or vehicle in the thermal escape box assay. Oxaliplatin is a commonly used chemotherapy drug that causes cold pain in both mice and humans (Krishnan et al., 2005). In line with our predictions, treatment with oxaliplatin resulted in significantly longer escape latencies at 5 °C compared to vehicle injected controls. Furthermore, treatment with the analgesic meloxicam significantly reduced escape latencies at 5 °C in mice that have received oxaliplatin treatment, compared to vehicle treated oxaliplatin-injected mice. Taken together, our results support the use the thermal escape box in preclinical pain studies to analyze the motivation and affective aspects of thermal pain and how these are affected by treatment with analgesics.

### 4.4. Assay limitations and experimental design suggestions for use

As with all behavioral assays, there are experimental design

considerations and limitations to the use of the thermal escape box. Our data caution against habituating mice to the thermal escape box apparatus and repeat testing on the same day. We recommend temperature testing orders follow a least-to-most noxious sequence, as in experiments were 5 °C preceded 18 °C (see Figs. 2, 3, 6) escape latencies at 18 °C tended to be longer (see Figs. 8–10 for comparison), though our experimental design does not allow for a direct statistical comparison of this observation. We found that repeat testing on different days separated by 24 h resulted in a significant increase in escape latency at 5 °C and 18 °C, but not at 30 °C or 52 °C. We predict that lengthening the intertrial testing window (i.e., to 72 h or more) would prevent the effects of repeat testing on escape latencies; however, this can be strain and paradigm specific. We recommend experimenters determine the appropriate intertrial testing window for their specific experimental context. Finally, as with all behavioral assays, performance in the thermal escape box will be impacted by the mouse's innate anxiety, which can be influenced by the genetic background of the strain. We recommend only conducting within strain comparisons in the thermal escape box.

### 4.5. Conclusion

Collectively, our data demonstrate that the thermal escape box is a new thermosensory behavioral paradigm that is useful for examining both physiological temperature-sensing and thermal pain. Future experiments are needed to validate the assay across different pain models and in other model organisms, such as rats.

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## CRediT authorship contribution statement

**Jacquelyn R. Dayton:** Formal analysis, Investigation, Validation, Visualization, Writing – review & editing. **Jose Marquez:** Formal analysis, Investigation, Validation, Writing – review & editing. **Alejandra K. Romo:** Formal analysis, Writing – review & editing. **Yi-Je Chen:** Investigation, Writing – review & editing. **Jorge E. Contreras:** Funding acquisition, Resources, Supervision, Writing – review & editing. **Theanne N. Griffith:** Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Theanne N. Griffith has assigned a provisional patent to the University of California related to the described behavioral assay. Other authors report no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynpai.2024.100155.

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