



# How two sesquiterpenes drive horse manure rolling behavior in wild giant pandas

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## Abstract

In this work, we discussed and counter-commented Paul J. Weldon's comments on our recent paper (Zhou et al. *Proc Natl Acad Sci USA* 117:32493, 2020a), where we reported that BCP/BCPO (beta-caryophyllene/caryophyllene oxide) in fresh horse manure is sufficient to drive manure rolling behavior (HMR) in giant panda and attenuate the cold sensitivity of mice by directly targeting and inhibiting transient receptor potential melastatin 8 (TRPM8), an archetypical cold-activated ion channel of mammals. The main question we arise in this response is: "which is the reasonable target of BCP/BCPO? Parasites or TRPM8?" Based on the knowledge of TRPM8-mediated cooling sensation, interaction between BCP/BCPO and TRPM8, BCP/BCPO concentration in horse manure samples, correlation between HMR frequency and habitat temperature, insecticidal activity of BCP/BCPO and thermal ecology of parasites, we prefer a simple idea that BCP/BCPO-induced TRPM8 antagonism bestows the wild giant pandas with cold tolerance at low-ambient temperatures. Compared with the speculation of insecticidal activity induced by HMR behavior, our study provided a comprehensive mechanism to confirm a physiological target of BCP/BCPO during the highly cold-correlated behavior.

**Keywords** Giant panda · Horse manure rolling · TRPM8 · Temperature

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## TRPM8 function in cold sensation

TRPM8, a tetrameric ion channel, was identified in cool-sensing thermoreceptive sensory neurons (Diver et al. 2019; McKemy et al. 2002; Nilius and Voets 2007; Yin et al. 2018). For the expression and distribution of the channel, Takashima et al. and Dhaka et al. genetically engineered mice to express EGFP in axons containing TRPM8 channels and indicated that fibers with this channel diffusely innervate the skin and oral cavity, terminating in peripheral zones that contain nerve endings mediating distinct perceptions of innocuous cool, noxious cold, and first- and second-cold pain (Dhaka et al. 2008; Takashima et al. 2007). It is also clear that genetic ablation of TRPM8 resulted in a marked decrease in cold-induced responses (Bautista et al. 2007; Colburn et al. 2007; Dhaka et al. 2007) or did not respond to innocuous or noxious cold temperatures (Knowlton et al. 2013). Consistently, knock-in mice expressing the penguin's TRPM8, a channel orthologous with low cold sensitivity, exhibited remarkable tolerance to cold (Yang et al. 2020). In pharmacology, the cooling compounds such as menthol, icilin, or others have been used long before it became clear that

TRPM8 mediates the responses to these chemical compounds that mimic cold temperatures (Chuang et al. 2004; Eccles 1994; Peier et al. 2002; Xu et al. 2020). In the contrast, TRPM8 antagonists are effective in the treatment of cold allodynia (Calvo et al. 2012). Therefore, BCP/BCPO directly inhibit cold- and ligand-induced TRPM8 activation, which is considered to cause a significant decrease in cold sensitivity of giant pandas, as seen in mice.

## Dose-dependence of BCP/BCPO-TRPM8 interaction

As illustrated in our data, the half-inhibitory concentration ( $IC_{50}$ ) value of BCPO is smaller than 10  $\mu$ mol for both cold and ligand activation. Although the authors did not test the exact concentration of BCP/BCPO in each horse manure sample, we can roughly provide an estimate to address this issue. The concentration of C14 fatty acids in fresh horse manure (around 100  $\mu$ g/g) has been reported (Kimura 2001), which comprised ~3% according to our GC–MS results. Therefore, the concentration of BCP/BCPO (~7%) was around 230  $\mu$ g/g, suggesting that the solution concentration was greater than 1000  $\mu$ mol. Even a slight fluctuation of C14 fatty acids should be considered, the concentration of BCP/BCPO should be greater than 100  $\mu$ mol, which we used in the rodent models. In another word, the concentration of BCP/BCPO is sufficient to block TRPM8 via HMR behavior.

## Correlation between HMR frequency and habitat temperature

Of note, both the frequency and duration of HMR events were increased in cold ambient temperatures, while no HMR events were recorded when the habitat temperatures were higher than 20 °C. In cold temperatures that promote TRPM8 activation, BCP/BCPO provided by HMR behavior is considered to strikingly suppress the channel opening. Coincidentally, host-parasite system also exhibits pronounced seasonal variation in infection prevalence (Leal et al. 2020; Zhou et al. 2020b). For the dynamics of tick population at all growth stages, peaks of larva, nymph, and adult are not expected to show up ranging from November of the first year to the March of the next year (Winter et al. 2021). This period, however, is the unique observation window of HMR events. Together, the recorded frequency and duration of HMR events nicely correlate with decreased habitat temperature.

## Insecticidal activity of BCP/BCPO

It is a common activity that essential oil of plants or some chemical surrogates are able to elicit lethal function or repellent activity against insects. To our knowledge, however, there is no solid evidence to show the repellent activity of BCP/BCPO against ticks or other ecto-parasites. Although BCP/BCPO have been reported that elicits antagonism to three agricultural pests with  $LD_{50}$  values of micro- to milli-molar range (Ma et al. 2020), little information can be interpreted to estimate the activity against intestinal or ecto-parasites.

## The hypothesis about defensive anointing

The authors believe the defensive use of chemical compounds may be employed by some animals, anointing behavior observed in non-human primates, for example (Falotico et al. 2004, 2007), to repel ecto-parasites. In the case of HMR behavior observed in giant pandas, at least four more tasks should be accomplished to support the possibility of defensive anointing: (1) identification of ecto-parasites in wild giant pandas; (2) dose dependence and molecular mechanism of BCP/BCPO for antagonizing each ecto-parasite; (3) efficiency of BCP/BCPO application in infected animal model; (4) correlation between HMR events and dynamics of the parasitic infection.

Lastly, the authors would like to clarify that the construction of the ancient trade roads makes panda-dunghill interaction stable and easy to be observed. The ancient trade routes for connecting Shu kingdom and Chang'an (referred to Xi'an, the former capital city in the history) made the captive horses for good transit a stable member in the present panda habitat for thousands of years, thereby made horse's dunghill and its odor regular to the population of QIN pandas. Therefore, it is unlikely a novel odor (snake scent gland secretions for example) to QIN pandas.

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## Declarations

**Conflict of interest** The authors has no relevant animal ethics or competing interests to disclose.

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