***Original Research Article***

**Comparative study of Stainless Steel and Brass Metal for Effectiveness of Freeze Branding in Sahiwal Cattle**

**ABSTRACT**

The study was conducted on 40 Sahiwal cattle of age group from 0 to 12 months. Cattles were divided into two groups (20 each). Stainless steel and brass metals were cooled in liquid nitrogen until frosting appeared, then firmly applied to the skin. In 0 to 6 months age group, exposure times were 5, 7, 9, and 11 seconds allotted to each of 5 animals. In 6 to 12 months group, exposure times were 8, 11, 14 and 17 seconds allotted to each of 5 animals. Immediately after freeze branding, several changes occurred on the animals skin. In the 0-6 months age group, skin thawing had faster at 5 and 11 seconds in stainless steel and at 11 seconds in brass. Development of oedema observed quicker at 11 seconds for both metals. Skin dried significantly faster at 7-9 seconds of exposure times in both metals. Scab formation and its persistency required fewer days at 7 seconds in stainless steel and brass. White hairs appeared faster at 5 and 9 seconds with stainless steel, with 100% legibility at 7 seconds. In the age group of 6-12 months, skin thawed fastest at 17 seconds in stainless steel and at 8 seconds in brass. Development of oedema observed quicker at 8 seconds in both metals. Significantly lesser days required for dryness of skin at 8 seconds of exposure time in both the metals. Scab formation and its persistency required fewer days at 8 seconds in stainless steel and 11 to 8 seconds in brass. White hair appeared faster at 8-11 seconds, with 80-100% legibility at 11 to 8 seconds for both metals. In conclusion, for 0-6 months, 5-7 seconds was recommended for stainless steel, 6-12 months, both metals can be used at 8-11 seconds.

***Keywords:*** *Sahiwal, freeze branding, white hairs, stainless steel, brass*

**1. INTRODUCTION**

Animal identification is an essential aspect of livestock management, involving the responsibility of unique markers or identifiers to individual animals. Marking, identifying, and registering livestock in Romania are regulated by normative acts (Bowling et al., 2008). Several methods are currently used to identify animals, though none are entirely flawless or foolproof (Nandanwar et al., 2015 and Mishra et al., 2005). A commonly used identification method across all species is ear tagging. Ear tags are easy to apply, adaptable to various weather conditions, and relatively inexpensive, making them the primary choice for initial identification in new dairy operations. However, their effectiveness is compromised as they can become smeared with mud and manure, reducing readability. One of these methods is ear notching, which is mostly used to identify pigs. The tattoos cannot be read without catching the head of animal (Hall *et al*., 2004) and is a biggest disadvantage of this method. Neck chains, paint mark and photographs are another temporary method used for short term marking of livestock species. Among the methods mentioned above, some limitations have been observed. Despite this, hot branding remains widely practiced in both organized and unorganized herds across India (Nandanwar et al., 2017b). This practice has been strongly criticized by the Society for the Prevention of Cruelty to Animals. This method creates a permanent mark but is extremely painful (Tucker et al., 2014). Over time, hair growth can obscure the scar, requiring repeated branding. Electronic identification is a promising solution, but its high cost limits adoption. Implementing it in rural areas or under field conditions remains a challenge. Freeze branding is a widely accepted livestock identification method, especially in developed countries. Freeze branding method of identification should be easy to read legible mark visible from a distance (30 feet (Farrell, 1966)), simple to apply, permanent, durable, cost-effective, cause minimal damage to the hide (Adcock et al., 2018) and is less painful than hot branding (Lay et al., 1992a and Schwartzkopf-Genswein et al., 1997). It is commonly used for marking large mammals (Farrell et al., 1978; Newton, 1978). Given the numerous advantages of freeze branding, it is essential for livestock owners, commercial dairy farmers, and the scientific community to prevent cases of forgery. Freeze branding experiments have primarily been conducted on exotic breeds, with no documented evidence for indigenous breeds such as Sahiwal. Additionally, there is no established data on the appropriate exposure time for freeze branding in Sahiwal cattle. The Sahiwal, one of India's best dairy breeds, has a red coat colour therefore the white mark/hair appeared on the skin will remain permanent lifelong (Bello et al., 2020) and legibility of white hairs was easily readable from distance. The present study was observed the best exposure time of different metals for effectiveness of freeze branding in Sahiwal.

**2. MATERIAL AND METHODS**

**2.1 Location and Place of Work**

The Bull Mother Experimental Farm, Anjora, Durg, is located at an elevation of 317 meters above mean sea level, with coordinates 21.11° latitude and 81.17° longitude. During summer, the maximum temperature rises to 45°C, while in winter, it drops to 10°C. The average annual rainfall is approximately 1071.16 mm.

**2.2 Selection of Animals**

The present study was conducted on 40 Sahiwal cattle of different age groups. The cattle were divided into two groups, each consisting of 20 animals (Table 1). Their ages ranged from 0 month to 12 months (Hall et al., 2004).

**2.3 Method of Freeze Branding**

Branding metals, such as stainless steel and brass, are essential instruments that facilitate the transfer of the required temperature to the animal's skin. While previous studies (Farrel et al., 1966 and Whitter et al., 1993) have assessed copper, aluminium, stainless steel and bronze metals as a branding material, the present study focuses on comparing freeze branding using stainless steel and brass. The metals were molded into an "I" shape, with the width and depth (face to back) of the branding instrument remaining the same for all age groups. However, the length and weight varied according to the age group of the animals (Bath et al., 1981). Liquid nitrogen was used as a refrigerant, maintaining a temperature of approximately -196°C while remaining in a liquid state (Key et al., 1977; Wagner et al., 2000). The frosted branding metal was quickly applied to the cattle's skin with firm pressure, ensuring it remained in place without slipping. There is no evidence of exposure time for freeze branding in Sahiwal, so we have taken different exposure time to perform the experiment In case of 0 to 6 months category, the exposure varied from 5 to 12 seconds with an interval of 2 seconds, hence, four different exposure time i.e. 5, 7, 9, 11 seconds were allotted to each of 5 animals of this group. In case of 6 to 12 months category, the exposure varied from 8 to 17 seconds with an interval of 3 seconds, hence, four different exposure time i.e. 8, 11, 14, 17 seconds were allotted to each of 5 animals of this group (Table 1). Therefore, this study aimed to observe the series of events after freeze branding i.e. thawing of skin, development of oedema, duration of oedema, dryness of skin, scab formation and its persistency, appearance of white hairs and legibility of white hairs and develop a scoring system for selecting the most suitable metal at different exposure times across various age groups. The events that occur after freeze branding are assessed visually.

**Table 1: Different exposure time proposed for different age group**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Age**  **( months)** | **No. of Animal** | | **Exposure Time (seconds)** | |
| **Stainless Steel** | **Brass** |
| 0-6 | 20 | 5 | 5 | 5 |
| 5 | 7 | 7 |
| 5 | 9 | 9 |
| 5 | 11 | 11 |
| 6-12 | 20 | 5 | 8 | 8 |
| 5 | 11 | 11 |
| 5 | 14 | 14 |
| 5 | 17 | 17 |

**2.4 STATISTICAL ANALYSIS**

In this investigation, evaluation of the effectiveness of branding at different exposure times, a one-way analysis of variance (ANOVA) (Snedecor and Cochran, 1994) was used. Duncan's Multiple Range Test (DMRT) (Steel and Torrie, 1984) was applied for further comparison of the results.

**3. RESULTS and DISCUSSION**

**3.1 The thawing of skin**

In the 0–6 months age group, the average thawing time of the skin after freezing was 1.68±0.13 minutes for stainless steel and 1.73±0.14 minutes for brass metal (Table 2). For stainless steel, the shortest thawing time of 1.5±0.22 minutes was observed at 5 and 11 seconds of exposure. In contrast, for brass metal, the minimum thawing time was recorded at 11 seconds of exposure. There was no significant difference in thawing time between stainless steel and brass metals for their respective exposure durations. This suggests that skin thawing occurred more quickly at 5 and 11 seconds (1.5±0.22 minutes) for stainless steel and at 11 seconds (1.4±0.29 minutes) for brass, leading to faster skin healing compared to other exposure times. In the 6–12 months age group of Sahiwal cattle, the average thawing time of the skin after freezing was 1.45±0.1 minutes for stainless steel and 1.3±0.09 minutes for brass metal (Table 2). There was no significant difference in thawing time between stainless steel and brass metals for their respective exposure durations. The quickest thawing occurred at 8 seconds (1.1±0.1 minutes) for stainless steel and 17 seconds (1.1±0.1 minutes) for brass, leading to faster skin healing compared to other exposure times. Overall, the thawing time for all metal types ranged from a minimum of 1.1±0.1 minutes to a maximum of 1.7±0.2 minutes(Table 2). These findings closely align with Hooven (1968), who reported that skin thawing occurred within 2 minutes in cattle. Additionally, Bertram et al. (2006) also observed that skin thawed within a few minutes.

**Table 2: Mean time of thawing of skin using various metals of same dimension in a given exposure time**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age Group | **0 to 6 months** | | | | Overall | **6 to 12 months** | | | | Overall |
| **Exposure Time**  (Seconds) | 5 | 7 | 9 | 11 |  | 8 | 11 | 14 | 17 |  |
| **Stainless steel** | 1.5±0.22 | 2±0.27 | 1.7±0.3 | 1.5±0.22 | 1.68±0.13 | 1.5±0.22 | 1.5±0.22 | 1.7±0.2 | 1.1±0.1 | 1.45±0.1 |
| **Brass** | 1.8±0.37 | 1.8±0.19 | 1.9±0.24 | 1.4±0.29 | 1.73±0.14 | 1.1±0.1 | 1.4±0.19 | 1.3±0.2 | 1.4±0.24 | 1.3±0.09 |

**3.2 Development of oedema**

In the 0–6 months age group of Sahiwal cattle, the average time for the onset of oedema following freezing was 12.98±0.74 minutes for stainless steel and 11.34±0.38 minutes for brass metal (Table 3). Oedema development occurred more quickly at 9 and 11 seconds of exposure (11.8±0.66 and 9.8±1.16 minutes, respectively) compared to 7 seconds (16±1.68 minutes), showing a significant difference (P<0.01). Overall, a downward trend was observed in the time required for oedema development as exposure time increased in this age group. For stainless steel, the time required for oedema onset gradually decreased, except at 7 seconds of exposure, where it took 16±1.68 minutes. In the 6–12 months age group, the average time for oedema onset was 13.5±1.01 minutes for stainless steel and 14.05±0.97 minutes for brass metal (Table 3). In both metals, oedema development showed an increasing trend with longer exposure times. However, using brass metal, the oedema onset at 8, 11, and 14 seconds differed significantly (P<0.05) from that at 17 seconds. Overall, these findings are consistent with those of Bertram et al. (2006), who observed noticeable swelling in the branded area within minutes in dark coat colour animal. However, Torell et al. (2001) reported that the swelling pattern emerged within 5 to 10 minutes in Ranch animals.

**Table 3: Mean time for development of oedema using various metals of same dimension in a given exposure time**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age Group | **0 to 6 months** | | | | Overall | **6 to 12 months** | | | | Overall |
| **Exposure Time**  (Seconds) | 5 | 7 | 9 | 11 |  | 8 | 11 | 14 | 17 |  |
| **Stainless steel** | 14.3±0.49bc | 16±1.68c | 11.8±0.66ab | 9.8±1.16a | 12.97±0.74\*\* | 11.2±0.97 | 12.2±0.86 | 13±1.14 | 17.6±3.2 | 13.5±1.01 |
| **Brass** | 11±0.45 | 12.2±0.58 | 12.3±0.66 | 9.8±0.86 | 11.32±0.38 | 11.6±0.68a | 12±0.63a | 14.2±1.11ab | 18.4±2.94b | 14.05±0.97\* |

Values superscripted by different letters differed significantly from each other in a column \*\*P<0.01&\*P<0.05

**3.3 Duration of oedema**

An average duration time of oedema following freezing was observed to be 23.55±0.63 hours for stainless steel and 23.2±0.66 hours for brass metal (Table 4). This duration ranged from 21.2±1.46 hours (minimum) to 25.6±0.92 hours (maximum) in Sahiwal cattle aged 0 to 6 months. In the case of brass metal, the duration of oedema for an exposure time of 9 seconds (25.6±0.92 hours) was significantly different (P<0.05) from that of 5 and 11 seconds (21.4±1.20 and 21.4±1.36 hours, respectively). For the 6 to 12 months age group of Sahiwal cattle, the average duration time of oedema following freezing was 21.65±0.65 hours for stainless steel and 21.5±0.77 hours for brass metal (Table 4). The differences in oedema duration time across all exposure durations in this age group for both stainless steel and brass metals were non-significant. Similar findings were observed by Hooven (1968) who reported that marked oedema persist for 24 to 48 hours. However, Torell et al. (2001) reported that the skin was swollen for the period of 48 to 72 hours after freeze branding.

**Table 4: Mean time for duration of oedema using various metals of same dimension in a given exposure time**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age Group | **0 to 6 months** | | | | Overall | **6 to 12 months** | | | | Overall |
| **Exposure Time**  (Seconds) | 5 | 7 | 9 | 11 |  | 8 | 11 | 14 | 17 |  |
| **Stainless steel** | 24±1.1 | 24.8±0.58 | 24.2±1.36 | 21.2±1.46 | 23.55±0.63 | 21.4±0.75 | 21.6±0.68 | 20±1.67 | 23.6±1.63 | 21.65±0.65 |
| **Brass** | 21.4±1.20a | 24.4±0.74ab | 25.6±0.92b | 21.4±1.36a | 23.2±0.66\* | 19.8±1.46 | 21.6±1.29 | 22.8±1.46 | 21.8±2.01 | 21.5±0.77 |

Values superscripted by different letters differed significantly from each other in a column \*P<0.05

**3.4 Dryness of skin**

The average number of days required for skin dryness following freezing was 7.15 ± 0.86 and 8.05 ± 0.68 for stainless steel and brass metals, respectively (Table 5). In Sahiwal cattle, skin dried faster after freeze branding at 7 and 9 second exposure times, followed by 5 and 11second exposure times. For stainless steel, the exposure times of 7 and 9 seconds differed significantly (P<0.01) from those of 5 and 11 seconds. In brass metal, the 7 and 9 second exposure times differed significantly (P<0.05) only from the 11 second exposure time. At a 7 second exposure time, the duration of edematous swelling was longer, whereas skin dryness occurred more quickly (Table 5) in the 0 to 6 month age group using stainless steel. In Sahiwal cattle aged 6 to 12 months, the average time required for skin dryness was 9.6 ± 0.86 and 10.75 ± 0.98 days for stainless steel and brass metals, respectively. For stainless steel, the number of days required for skin dryness at 8 and 11 second exposure times differed significantly (P<0.05) from that at 17 second exposure time (13 ± 1.64 days). Similarly, when brass metal was used, the 17 second exposure time (14.6 ± 2.i25 days) differed significantly (P<0.05) from the8 and 11 second exposure times (Table 5).For both metals, skin dryness occurred significantly (P<0.05) faster at shorter exposure times. Overall, it can be concluded that skin dryness was more pronounced with stainless steel, followed by brass. Hooven (1968) reported that the dryness of the skin took 9 to 13 days in animals after freeze branding; however, he studied only one metal in his experiment.

**Table 5: Mean time for dryness of skin using various metals of same dimension in a given exposure time**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age Group | **0 to 6 months** | | | | Overall | **6 to 12 months** | | | | Overall |
| **Exposure Time**  (Seconds) | 5 | 7 | 9 | 11 |  | 8 | 11 | 14 | 17 |  |
| **Stainless steel** | 9.8±1.74b | 4±0.32a | 4.8±1.11a | 10±1.41b | 7.15±0.86\*\* | 6.4±1.29a | 8±1.05ab | 11±1.38bc | 13±1.64c | 9.6±0.86\* |
| **Brass** | 8.2±1.24ab | 6.8±0.86a | 6.2±1.15a | 11±1.30b | 8.05±0.68\* | 8±1.7a | 8.6±1.54a | 11.8±0.86ab | 14.6±2.25b | 10.75±0.98\* |

Values superscripted by different letters differed significantly from each other in a column \*\*P<0.01&\*P<0.05

**3.5 Formation of scab and its persistency**

In Sahiwal, the average time for formation of scab and its persistency were 16.65±1.18 and 18.8±0.81 days for stainless steel and brass metals, respectively in 0-6 months of age (Table 6). In stainless steel metals, the time required for scab formation and its persistency at 11 seconds of exposure time differed significantly (P<0.01) from rest of the exposure time. This result indicated that more days were required for scab formation and its persistency at higher exposure time (11 seconds) whereas, in this age group of similar exposure time (11 seconds) the time required for development of oedema was significantly (P<0.05) lesser (9.8±1.16 minutes) (Table 6) in stainless steel metal. However, in brass metal no significant result was seen. In 6 to 12 months of age group, the average time required for formation of scab and its persistency in present investigation was found to be 19.4±0.73 and 20.3±0.87 days for stainless steel and brass metals, respectively (Table 6). In brass metal, a significant difference (P<0.05) were noticed between the time required for scab formation and its persistency for the exposure time of 11 and 17 seconds (Table 6). That means higher the exposure time (17 seconds) more days will be required for scab formation and its persistency. In stainless steel metals increasing trend for scab formation and its persistency were seen in 6 to 12 months age group Sahiwal. However, these findings were in accordance with the findings of Bath et al. (1981) and Bertram et al. (2006) observed 3 to 4 weeks of time which is little higher than the required days for formation of scab and its persistency.

**Table 6 Mean timefor scab formation and its persistency using various metals of same dimension in a given exposure time**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age Group | **0 to 6 months** | | | | Overall | **6 to 12 months** | | | | Overall |
| **Exposure Time**  (Seconds) | 5 | 7 | 9 | 11 |  | 8 | 11 | 14 | 17 |  |
| **Stainless steel** | 16.8±2.18a | 11.8±0.2a | 15.6±2.01a | 22.4±1.78b | 16.65±1.18\*\* | 17±1.87 | 18±0.55 | 21±1.14 | 21.6±1.12 | 19.4±0.73 |
| **Brass** | 20±1.26 | 16.8±1.39 | 17.4±1.69 | 21±1.70 | 18.8±0.81 | 18.6±0.98a | 18±1.3a | 20.4±1.12ab | 24.2±2.18b | 20.3±0.87\* |

Values superscripted by different letters differed significantly from each other in a column \*\*P<0.01&\*P<0.05

**3.6 Appearance of white hairs**

The average time for the appearance of white hairs on the skin was **46.63 ± 1.97** days for stainless steel and **50.93 ± 1.3** days for brass metals in 0-6 months of age group (Table 7). A shorter time was observed for the appearance of white hairs at **5 and 9 second** exposure times (**44.75 ± 3.79**and**45.75 ± 5.57** days, respectively) using stainless steel. However, all values were **non-significantly different** across metals and exposure times in this study. The present findings align with those of **Sherwin et al. (2002),** who reported that the regrowth of white hairs occurred within **22 to 60 days** (average **36 days**). Similar finding reported by Nandanwar **et al. (2017b) suggested that white hairs appeared on the skin of cattle within 41 to 51 days using stainless steel metal.** In the **6 to 12 month** age group, the average time required for the appearance of white hairs following freezing was **46.78 ± 1.96** and **50.2 ± 1.42** days for stainless steel and brass metals, respectively (Table 7). In this study, a relatively shorter time was required for white hair appearance at **8 to 11 second** exposure times using both metals. These results (Table 7) are in agreement with the findings of **Bertram et al. (2006),** who observed growth of white hairs on the skin of animals appeared within six weeks. Similarly, Nandanwar **et al. (2017a)** who reported that white hair growth appeared on skin of Sahiwal cattle within **seven weeks at 8 seconds of exposure time in brass metal after freeze branding.**

**Table 7: Mean time for appearance of white hairs using various metals of same dimension in a given exposure time**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age Group | **0 to 6 months** | | | | Overall | **6 to 12 months** | | | | Overall |
| **Exposure Time**  (Seconds) | 5 | 7 | 9 | 11 |  | 8 | 11 | 14 | 17 |  |
| **Stainless steel** | 44.75±3.79 | 47.6±3.33 | 45.75±5.57 | 49.50±2.50 | 46.63±1.97 | 45.6±5.03 | 45.5±3.18 | 48±2.08 | 50.5±1.50 | 46.78±1.96 |
| **Brass** | 49±1.83 | 50.66±2.33 | 48.75±3.94 | 55.25±1.75 | 50.93±1.3 | 47.2±2.4 | 49.75±1.75 | 56.66±2.40 | 49.66±3.28 | 50.2±1.42 |

**3.7 Legibility of freeze brand**

The frequency of legible white hairs on the animals using for stainless steel metal were 80, 100, 80 and 40 percent, respectively for 5, 7, 9 and 11 seconds of exposure time. However, 80, 60, 80 and 80 per cent of legible white hairs were observed using brass metal for above mentioned exposure time used in 0 to 6 months of age group of Sahiwal (Table 8). 100 percent legibility was seen in 7 seconds of exposure time using stainless steel metal. Considering this level of acceptability, 80 per cent legibility was seen at 5 and 9 seconds of exposure time in stainless steel brand. Overall, in this age group, the exposure time recommended for stainless steel and brass metals are 5 to 7 seconds and 5 seconds, respectively (Table 8). In age group of 6 to 12 months, 100 per cent legibility of white hairs was seen at 8 seconds of exposure time, while it was 80 per cent at 11 seconds of exposure time. In stainless steel and brass metal where 80 to 100 per cent legible white hairs were observed at 11 to 8 seconds of exposure time, respectively (Table 8). Therefore, in this age group (6 to 12 months) all the brands used could be recommended with an exposure time ranging from 8 to 11 seconds. Similar finding reported by Nandanwar **et al. (2017b) suggested that 100 percent legibility of white hairs at 7 seconds of exposure time using stainless steel metal in 0-6 months of age group in Sahiwal cattle.** Similarly, Nandanwar **et al. (2017b)** who reported that **100 percent legibility of white hairs at 8 seconds of exposure time using stainless steel metal in 6-12 months of age group in Sahiwal cattle.**

**Table 8: Mean value of frequency (in percentage) of legible white hairs using various metals of same dimension on the animals for given exposure time**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age Group | **0 to 6 months** | | | | Chi square | **6 to 12 months** | | | | Chi square |
| **Exposure Time**  (Seconds) | 5 | 7 | 9 | 11 |  | 8 | 11 | 14 | 17 |  |
| **Stainless steel (%)** | 80 | 100 | 80 | 40 | 5.064 | 100 | 80 | 60 | 40 | 4.758 |
| **Brass (%)** | 80 | 60 | 80 | 80 | 0.798 | 100 | 80 | 60 | 80 | 2.5 |

**4. CONCLUSIONS**

Present study found that white hairs was appeared faster at 5 and 9 seconds of exposure time, with 100 percent legibility of white hairs was observed at 7 seconds of exposure time using stainless steel metal in 0-6 months of age during freeze branding in Sahiwal cattle. Similarly, white hair appeared faster at 8 to 11 seconds of exposure time, with 80–100 percent legibility of white hairs was observed at 8 to 11 seconds for both metals in 6-12 months of age in Sahiwal cattle. In conclusion, for 0–6 months, 5 to 7 seconds is recommended for stainless steel. For the **0 to 6 month** age group, an exposure time of **5 to 7 seconds** is recommended for stainless steel metal and for **6 to 12 months of age group,** both metals used in this experiment can be recommended with an **exposure time of 8 to 11 seconds.**

**Disclaimer (Artificial intelligence)**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

**Competing Interests**

Authors have declared that no competing interests exist

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