

Hydrogel-Based Topical Delivery of Isoflurane in the Giant African Land Snail (*Achatina fulica*)

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Abstract

Anesthesia of terrestrial gastropods remains challenging, and conventional delivery routes may not consistently suppress *Achatina fulica*. This pilot study evaluated a hydrogel-based formulation as a topical delivery system for isoflurane and characterized associated behavioral and functional responses. Eleven adult *A. fulica* were evaluated using a randomized crossover design comprising untreated baseline, saline immersion, hydrogel alone, and hydrogel containing isoflurane. Behavioral, locomotor, withdrawal, mucus, and body tone responses were assessed at predefined time points using a structured ordinal scoring framework. At 5 min, animals receiving Gel-Isoflurane showed significantly higher scores across all assessed domains and the composite score than all control conditions ($P = 0.006$). Gel alone and saline immersion did not produce comparable multidomain suppression. After hydrogel removal, scores progressively decreased, and all animals returned to their predefined baseline state. Median recovery time was $66 \pm \text{min}$ (IQR, 57.5–72.5 min; range, 42–84 min). Interobserver reliability was high, with an intraclass correlation coefficient of 0.981 for the composite score. These findings provide preliminary evidence that hydrogel-mediated topical delivery can produce measurable and reversible functional changes following isoflurane exposure in *A. fulica*. Further studies are required to establish dose–response relationships, pharmacokinetics, physiological effects, and validity of species-appropriate anesthetic endpoints.

Introduction

Anesthesia is an important part of veterinary practice and experimental research because it facilitates immobilization and procedural handling while helping to minimize stress and support animal welfare during potentially painful or invasive procedures [1–3]. As attention to animal welfare has expanded beyond vertebrate species, the need for appropriate anesthetic or analgesic approaches in invertebrates has also received increasing attention [3–5]. Evidence-based guidance, however, remains limited for many invertebrate taxa, particularly with respect to effective drug protocols, anesthetic depth, and appropriate monitoring [6, 7]. The wide anatomical and physiological diversity of invertebrates further complicates this issue and can influence drug uptake, pharmacological responses, and suitable routes of administration [7–9].

Gastropods are a particularly diverse group, with more than 80,000 species inhabiting marine, freshwater, and terrestrial environments [10]. In snails, reliable anesthesia can be important because withdrawal and retraction into the shell can make handling and clinical procedures difficult. Much of the existing literature, however, has focused on immobilization, fixation, or euthanasia rather than reversible anesthesia in animals intended to recover [4, 11, 12]. In addition, apparent sedation or loss of movement does not necessarily indicate a consistent depth of general anesthesia or demonstrate analgesia [5, 8].

A wide range of chemical agents has been investigated for gastropod immobilization and anesthesia, including gases and volatile liquids that can be administered through an anesthetic chamber in terrestrial species or introduced into the water container in aquatic species [4, 9]. Immersion techniques

have commonly employed magnesium salts [13–15], ethanol [13, 16, 17], tricaine methanesulfonate (MS-222) [5, 18, 19], and essential oils such as menthol [20–22], eugenol [13, 23, 24], and clove oil [11, 25, 26]. Numerous other compounds, including injectable, local, and general anesthetic agents, have also been investigated experimentally [7, 14, 22, 27–33]. However, their efficacy varies considerably among species, and reductions in movement or apparent sedation do not necessarily indicate a consistent anesthetic state [5, 34, 35]. The mechanisms underlying these responses are also not always clear, with uncertainty regarding whether some agents produce anesthesia and analgesia or act primarily through neuromuscular effects [6, 7, 19]. Behavioral immobility alone may therefore be insufficient to distinguish sedation, immobilization, and anesthesia, emphasizing the need for species-appropriate assessment criteria [5, 6, 34].

Practical anesthesia of terrestrial gastropods, including the giant African land snail (*Achatina fulica*), can be particularly challenging and highly variable in effect [31, 35, 36]. Although immersion in ethanol or certain essential-oil preparations has produced successful anesthesia in some gastropod species [12, 16, 17], several commonly used anesthetic approaches have shown limited or inconsistent effects [11, 22]. In giant African land snails, attempts using alfaxalone, ketamine, ketamine–dexmedetomidine combinations, and different routes of administration failed to produce effective anesthesia consistently. Similarly, conventional chamber delivery of isoflurane did not produce clinically apparent sedation or anesthesia in the reported snails [31]. These findings illustrate the difficulty of translating anesthetic approaches that are effective in other invertebrates directly to terrestrial gastropods.

Immersion in volatile anesthetics such as halothane, isoflurane, and enflurane can reversibly suppress withdrawal responses in some aquatic mollusks [19, 37, 38]. However, conventional inhalational techniques may be less practical for pulmonate and/or terrestrial snails, because anesthetic exposure is dependent on a chamber with fresh gas inflow and waste gas scavenging for continued contact with the anesthetic [5, 37, 39]. In contrast, clinical procedures generally require removal of the animal from the induction chamber. This can result in fluctuating anesthetic depth and may complicate control of exposure and can increase environmental contamination by volatile agents [5, 6, 8]. The mucus-covered body surface and respiratory characteristics of terrestrial snails may also affect the consistency of anesthetic uptake.

An alternative delivery concept has been explored in amphibian anesthesia. Inhalational administration may be associated with variable induction and rapid recovery after removal from the chamber, particularly when pulmonary ventilation decreases during anesthesia [40–42]. Transcutaneous uptake can become increasingly relevant under these conditions [43]. Topical administration of liquid isoflurane and the use of viscous carriers have therefore been investigated as alternative approaches [44, 45]. In particular, formulations combining liquid isoflurane with a viscous, water-soluble carrier such as K-Y Jelly have been used to maintain contact between the anesthetic and the skin and to facilitate topical exposure [44, 45]. Removal of the formulation after induction can then be used to terminate further exposure; although dose titration may be less straightforward than with conventional inhalational administration [46]. This approach provides a conceptual basis for exploring hydrogel-mediated topical

delivery in terrestrial gastropods, where maintaining contact with the mucus-covered body surface may be important.

Hydrogels are widely used in topical drug delivery because they can alter the interaction between an active compound and the biological surface to which it is applied [47, 48]. Gel-based systems have been investigated extensively in human and veterinary medicine [49–51], but their use as carriers for volatile anesthetics has not previously been evaluated in terrestrial gastropods, to our knowledge. We therefore hypothesized that incorporating isoflurane into a hydrogel-based formulation would produce measurable and reversible changes in behavioral and functional responses in *A. fulica* while providing a practical topical delivery approach. Because reliably assessing of anesthetic responses is itself challenging in invertebrates, particularly when standard physiological monitoring is unavailable [5, 7, 8], we also used a structured framework incorporating behavioral activity, locomotion, withdrawal responsiveness, mucus secretion, and body tone. These endpoints were considered together rather than relying on a single reflex, in line with previous gastropod studies [31, 37, 52].

The objective of this pilot study was therefore to evaluate an isoflurane-containing hydrogel as a topical delivery formulation in *A. fulica* and to characterize the associated behavioral and functional responses. The study compared the formulation with untreated baseline, saline immersion, and gel-only conditions and examined the temporal pattern of responses and recovery. The scoring framework was used as an exploratory functional assessment tool and was not intended to represent a validated measure of anesthetic depth.

Results

A total of 11 adult *Achatina fulica* were included in the study. Each animal underwent all four experimental conditions (Baseline, Immersion, Gel, and Gel-Isoflurane) using a repeated-measures crossover design, with a three-day washout period between consecutive experimental sessions. All animals completed every experimental session, and we excluded no observations from the analyses. Repeated measurements obtained during the Gel-Isoflurane condition were subsequently analyzed to characterize the temporal progression of anesthesia.

Consensus dataset

Observer ratings were aggregated using the median score for each animal, experimental condition, and observation time point, generating a consensus dataset for all subsequent analyses. This consensus dataset consisted of 88 observations (11 animals × 8 observation conditions), with no missing values.

Interobserver reliability

Agreement among the four independent observers was consistently high across all five behavioral domains (Table 1). Fleiss' kappa values ranged from 0.755 for body tone to 0.976 for withdrawal

responses, indicating substantial to almost perfect agreement. Likewise, Krippendorff's alpha coefficients ranged from 0.835 to 0.996, confirming excellent interobserver consistency across all scoring domains. All Fleiss' kappa statistics were highly significant ($P < 0.001$). The composite behavioral score demonstrated excellent interobserver reliability (ICC = 0.981, 95% CI: 0.973–0.987, $p < 0.001$). Pairwise weighted Cohen's kappa coefficients demonstrated similarly high agreement between every observer pair for all behavioral domains (**Supplementary Table S1**), further supporting the robustness and reproducibility of the developed framework.

Table 1

Interobserver reliability of the framework assessed using Fleiss' kappa and Krippendorff's alpha.

Domain	Subjects	Raters	Fleiss' κ	κ interpretation	Krippendorff's α	α interpretation
Behavioral score	88	4	0.790	Substantial	0.911	Almost perfect
Locomotion score	88	4	0.769	Substantial	0.918	Almost perfect
Withdrawal score	88	4	0.976	Almost perfect	0.996	Almost perfect
Mucus score	88	4	0.868	Almost perfect	0.890	Almost perfect
Body tone score	88	4	0.755	Substantial	0.835	Almost perfect
Footnote: Subjects = animal–timepoint observations; Raters = independent observers. Fleiss' κ and Krippendorff's α indicate interobserver agreement. Conventional thresholds were used to interpret agreement (0.61–0.80, substantial; ≥ 0.81 , almost perfect). All Fleiss' κ values were significant at $P < 0.001$.						

Comparison of experimental conditions at 5 minutes

Behavioral performance differed significantly among the four experimental conditions at 5 minutes after treatment for all evaluated domains (Table 2, Fig. 1). Friedman tests demonstrated significant overall effects for behavioral score ($\chi^2 = 33.0$, $df = 3$, $P < 0.001$, Kendall's $W = 1.00$), locomotion score ($\chi^2 = 28.6$, $df = 3$, $P < 0.001$, Kendall's $W = 0.868$), withdrawal score ($\chi^2 = 33.0$, $df = 3$, $P < 0.001$, Kendall's $W = 1.00$), and body tone score ($\chi^2 = 31.7$, $df = 3$, $P < 0.001$, Kendall's $W = 0.960$), indicating large treatment effects for all behavioral domains.

Post hoc Wilcoxon signed-rank tests with Holm correction revealed a consistent pattern across these four domains. Animals treated with Gel-Isoflurane exhibited significantly higher behavioral, locomotion, withdrawal, and body tone scores than animals in the Baseline, Gel, and Immersion groups (all adjusted $P = 0.006$) (Fig. 3.c). In contrast, no significant differences were detected among the Baseline, Gel, and Immersion groups for any of these domains (all adjusted $P \geq 0.750$).

A slightly different pattern was observed for mucus secretion. Although the overall treatment effect remained significant (Friedman $\chi^2 = 27.0$, $df = 3$, $P < 0.001$, Kendall's $W = 0.818$), pairwise comparisons demonstrated that, in addition to the markedly increased mucus secretion observed in the Gel-Isoflurane group compared with all other treatments (adjusted $P = 0.006$) (Fig. 3.d), the Immersion group also showed significantly higher mucus secretion scores than the Baseline group (adjusted $P = 0.023$). However, the difference between the Gel and Immersion groups was no longer significant after Holm correction (adjusted $P = 0.062$).

The composite score differed significantly among the four treatment groups at 5 min after intervention (Friedman test: $\chi^2(3) = 23.09$, $P < 0.001$), with a large effect size (Kendall's $W = 0.701$). Post hoc pairwise comparisons demonstrated that the Gel-Isoflurane group had significantly higher composite scores than the Baseline, Gel, and Immersion groups (adjusted $P = 0.006$ for all comparisons). No significant differences were detected among the Baseline, Gel, and Immersion groups (all adjusted $P > 0.05$).

Table 2

Pairwise comparisons of domains among experimental conditions at 5 minutes after treatment.

Domain	Overall Friedman test	Significant pairwise comparisons (Holm-adjusted)
Behavioral score	$\chi^2 = 33.0$, $df = 3$, $P < 0.001$, $W = 1.00$	Gel-Isoflurane vs Baseline ($P = 0.006$); Gel-Isoflurane vs Gel ($P = 0.006$); Gel-Isoflurane vs Immersion ($P = 0.006$)
Locomotion score	$\chi^2 = 28.6$, $df = 3$, $P < 0.001$, $W = 0.868$	Gel-Isoflurane vs Baseline ($P = 0.006$); Gel-Isoflurane vs Gel ($P = 0.006$); Gel-Isoflurane vs Immersion ($P = 0.006$)
Withdrawal score	$\chi^2 = 33.0$, $df = 3$, $P < 0.001$, $W = 1.00$	Gel-Isoflurane vs Baseline ($P = 0.006$); Gel-Isoflurane vs Gel ($P = 0.006$); Gel-Isoflurane vs Immersion ($P = 0.006$)
Mucus score	$\chi^2 = 27.0$, $df = 3$, $P < 0.001$, $W = 0.818$	Gel-Isoflurane vs Baseline ($P = 0.006$); Gel-Isoflurane vs Gel ($P = 0.006$); Gel-Isoflurane vs Immersion ($P = 0.006$); Immersion vs Baseline ($P = 0.023$)
Body tone score	$\chi^2 = 31.7$, $df = 3$, $P < 0.001$, $W = 0.960$	Gel-Isoflurane vs Baseline ($P = 0.006$); Gel-Isoflurane vs Gel ($P = 0.006$); Gel-Isoflurane vs Immersion ($P = 0.006$)
Composite score	$\chi^2 = 23.1$, $df = 3$, $P < 0.001$, $W = 0.701$	Gel-Isoflurane vs Baseline ($P = 0.006$); Gel-Isoflurane vs Gel ($P = 0.006$); Gel-Isoflurane vs Immersion ($P = 0.006$)
Footnote: W = Kendall's coefficient of concordance; P values for pairwise comparisons were adjusted using the Holm method. Baseline, untreated condition; Immersion, saline immersion; Gel, hydrogel vehicle; Gel-Isoflurane, isoflurane-containing hydrogel.		

Temporal response during Gel-Isoflurane

To characterize the temporal response following Gel-Isoflurane exposure, behavioral performance was evaluated at 5, 10, 20, 30, and 45 min after exposure to the Gel-Isoflurane formulation (Table 3, Fig. 2). Friedman tests demonstrated significant time-dependent changes in all behavioral domains, including behavioral score ($\chi^2 = 38.5$, $df = 4$, $P < 0.001$, Kendall's $W = 0.874$), locomotion ($\chi^2 = 40.9$, $df = 4$, $P < 0.001$,

W = 0.930), mucus secretion ($\chi^2 = 37.5$, df = 4, $P < 0.001$, W = 0.853), body tone ($\chi^2 = 38.5$, df = 4, $P < 0.001$, W = 0.876), and the composite score ($\chi^2 = 43.6$, df = 4, $P < 0.001$, W = 0.991), indicating substantial temporal effects for all outcomes. Withdrawal responses also changed significantly over time ($\chi^2 = 22.2$, df = 4, $P = 0.00018$, W = 0.504), although the effect size was smaller than for the other behavioral domains.

Post-hoc Wilcoxon signed-rank tests with Holm correction demonstrated significant temporal changes in behavioral, locomotor, mucus, body tone, and composite scores following Gel-Isoflurane exposure, with scores generally decreasing over the observation period. Behavioral scores differed significantly between multiple time points. Locomotion and body tone scores showed a similar pattern, with significant differences observed primarily between earlier and later time points. Mucus scores also differed significantly across several time intervals, whereas no pairwise comparison for withdrawal scores remained significant after Holm adjustment despite a significant overall Friedman test. The composite score showed the most extensive pattern of pairwise differences, with significant changes across nearly all assessed intervals (**Supplementary Table S2**). Overall, the temporal pattern was characterized by marked suppression during the early and intermediate observation periods followed by a gradual decline in scores toward baseline after termination of topical exposure. Although substantial suppression was evident by approximately 20–30 min, composite scores continued to change significantly through 45 min, indicating that the response was not fully stable during the late observation period.

Recovery time

Recovery time was evaluated only in the Gel-Isoflurane group because no anesthetic state was induced in the Baseline, Immersion, or Gel groups. Isoflurane exposure was terminated after 5 min by completely removing the gel from the animal's body surface. Recovery time was therefore calculated as the interval between gel removal and the return to pre-exposure baseline values. The median recovery time was 66 min (IQR, 57.5–72.5 min; range, 42–84 min).

Table 3
Friedman test results for time-course analysis within the Gel-Isoflurane group.

Domain	Friedman χ^2	df	P value	Kendall's W	Effect size
Behavioral score	38.50	4	< 0.001	0.874	Large
Locomotion score	40.91	4	< 0.001	0.930	Large
Withdrawal score	22.18	4	< 0.001	0.504	Large
Mucus score	37.45	4	< 0.001	0.853	Large
Body tone score	38.54	4	< 0.001	0.876	Large
Composite score	43.64	4	< 0.001	0.991	Large
Footnote: Kendall's W = coefficient of concordance and effect-size measure for the Friedman test. Values of $W \geq 0.50$ were interpreted as indicating a large effect.					

Discussion

The present pilot study provides preliminary evidence that an isoflurane-containing hydrogel can produce measurable and reversible changes in several behavioral and functional responses in the giant African land snail (*Achatina fulica*). At 5 min, Gel-Isoflurane produced significantly higher behavioral, locomotor, withdrawal, mucus, body tone, and composite scores than the baseline, saline-immersion, and gel-only conditions; whereas gel alone did not produce a comparable multidomain response. After removal of the hydrogel, scores declined over time, and all animals returned to their predefined baseline state. These findings support the feasibility of the tested topical formulation and indicate that the observed response was associated with the isoflurane-containing treatment rather than with the gel vehicle or brief saline immersion.

One possible explanation for the observed response is the prolonged contact between the isoflurane-containing formulation and the body surface. The hydrogel provided a viscous carrier that remained in contact with the snail during the 5-min exposure period, which may have increased the opportunity for isoflurane transfer compared with a more rapidly evaporating liquid preparation. However, the present study did not measure drug residence time, tissue penetration, or local isoflurane concentrations. Prolonged contact should therefore be regarded as a possible contributor to the observed response rather than as a demonstrated pharmacokinetic property of the formulation. More generally, hydrogel and other viscous formulations have been used to increase the duration of topical contact between pharmaceutical compounds and biological surfaces [53–55], providing a conceptual basis for the delivery approach used here.

The findings are also relevant to previous attempts to anesthetize *A. fulica*. In a clinical report involving 12 giant African snails undergoing diagnostic biopsy, several approaches, including alfaxalone, ketamine, ketamine–dexmedetomidine, and inhalational isoflurane, did not consistently produce a suitable

anesthetic state. In particular, 5% isoflurane administered through an induction chamber for 20 min did not result in detectable sedation or anesthesia [31]. By comparison, isoflurane produced concentration-dependent suppression of withdrawal responses in the aquatic gastropod *Lymnaea stagnalis*, with an estimated ED50 of approximately 1.09% v/v; whole-body withdrawal was also reported to be more reproducible than tentacular withdrawal [37]. These differences suggest that the limited response observed with some terrestrial protocols may depend partly on exposure route and conditions of drug-tissue contact rather than on a lack of activity of isoflurane itself. The present study extends this literature by evaluating a topical hydrogel-based delivery approach in a terrestrial gastropod.

A previous study of 5% ethanol immersion in *A. fulica* provides a useful point of comparison. In that study, median induction and recovery times were approximately 25 and 50 min, respectively. Recovery was variable, however, and one animal died during immersion; mucus secretion and bubble production were each reported in 13.3% of animals [16]. The present approach differs in that the hydrogel was applied topically rather than used for whole-body immersion. For terrestrial gastropods, this may offer a different way to maintain anesthetic contact while avoiding immersion of the entire animal. Volatile anesthetics are otherwise commonly administered through an anesthetic chamber, whereas many agents used in aquatic gastropods are introduced directly into the surrounding water [4, 9]. Whether these differences in delivery route translate into differences in efficacy, safety, induction, or recovery remains to be determined in standardized comparative studies.

The formulation strategy was conceptually informed by previous amphibian studies, in which topical or transcutaneous exposure to volatile anesthetics has been explored as an alternative to conventional inhalational administration. These studies have used viscous carriers such as K-Y Jelly to prolong skin contact with liquid isoflurane, with removal of the formulation used to terminate further exposure [44, 45]. The present study adapted this delivery principle to *A. fulica* rather than directly applying an amphibian protocol. The marked response observed here suggests that maintaining prolonged contact between a volatile anesthetic and the external body surface may be a transferable delivery concept. However, species-specific differences in integument, mucus production, respiratory physiology, metabolism, and anesthetic uptake may substantially affect its performance.

The temporal response observed following Gel-Isoflurane exposure further supports the reversibility of the observed functional changes. Behavioral, locomotor, withdrawal, mucus, body tone, and composite scores increased following exposure and subsequently decreased after removal of the hydrogel (Fig. 3). The decline in functional scores after hydrogel removal provides a descriptive indication of recovery from the observed treatment-associated effects. All assessed parameters returned to their predefined baseline values, with a median recovery time of 66 min (IQR, 57.5–72.5 min; range, 42–84 min). These values describe the recovery profile observed under the specific formulation and exposure conditions used here and should not be considered established recovery times for isoflurane anesthesia in *A. fulica*. Previous studies in other molluscan species, including *Lymnaea stagnalis*, *Aplysia californica*, and *Octopus vulgaris*, have reported different induction and recovery profiles following volatile-anesthetic exposure [19, 37, 38]. Such differences indicate that recovery may vary among species and experimental

conditions. Because systemic and tissue isoflurane concentrations were not measured in the present study, the observed recovery kinetics cannot be attributed to a specific pharmacokinetic process.

The temporal pattern was not uniform across the assessed functional domains. All six outcomes changed significantly over time after Gel-Isoflurane exposure, with the largest temporal effect observed for the composite score (Kendall's $W = 0.991$) and the smallest for withdrawal responsiveness ($W = 0.504$). Scores generally declined during the observation period after hydrogel removal, although the extent and timing of these changes differed between domains. Tentacular withdrawal was the last assessed response to return to baseline (Fig. 3.e-f). This finding is notable because whole-body withdrawal has been reported as a more reproducible anesthetic endpoint than tentacular withdrawal in *Lymnaea stagnalis*, and tentacular responses in that species recovered rapidly after being lost [37]. Differences between the two species may therefore reflect variation in the sensitivity or recovery characteristics of these reflexes. Snail withdrawal responses are associated with changes in neural activity following stimulation [12, 35, 56], but persistence or loss of a single reflex should not be interpreted on its own as a direct measure of anesthetic depth or nociception [7, 37]. The present findings instead support using multiple complementary functional responses when characterizing treatment-associated changes. The high interobserver reliability observed for the composite score (ICC = 0.981, 95% CI 0.973–0.987) indicates that the scoring procedure was reproducible among observers, but agreement between observers does not by itself establish the validity of the framework as a measure of anesthetic depth.

Mucus secretion may reflect several concurrent responses in terrestrial gastropods. Its amount, viscosity, and appearance can change after disturbance and may depend on the nature and intensity of the stimulus [57, 58]. Abundant or altered mucus secretion has also been described in *A. fulica* [16, 31], and other gastropods [11, 12, 19, 35, 59] during handling, anesthetic procedures, or exposure to potentially harmful stimuli. In the present study, mucus scores differed both between experimental conditions and over time, indicating that mucus secretion was responsive to the experimental procedures (Fig. 3.d). The biological meaning of this response remains uncertain, however, because handling, mechanical stimulation, anesthetic exposure, and environmental changes may all contribute [31]. Notably, mucus production is not unique to terrestrial snails and has also been reported during anesthetic or stressful procedures in other invertebrates, including abalones, slugs, and jellyfish [27, 60, 61]. Mucus secretion could also modify the hydrogel–surface interface and thereby affect topical exposure, although this possibility was not directly assessed. Similarly, the possibility that mucus contributed to isoflurane elimination remains speculative because isoflurane concentrations in mucus were not measured. Future studies combining quantitative mucus characterization with pharmacokinetic measurements may help clarify the relationship between mucus production and topical anesthetic exposure.

The lack of a comparable multidomain response in the gel-only and saline-immersion conditions helps distinguish the Gel-Isoflurane response from effects associated with the vehicle or brief immersion procedure. This distinction is relevant because handling, immersion, and mechanical stimulation can

independently alter locomotion, posture, and mucus secretion in gastropods. Under the conditions tested here, the stronger and more coordinated response observed with Gel-Isoflurane was therefore associated with the isoflurane-containing formulation rather than with the gel or saline treatment alone. From a practical perspective, topical hydrogel delivery may be useful in experimental settings where reduced movement is required without whole-body immersion. However, whether the approach is suitable for clinical procedures remains unresolved and will require studies addressing safety, dose, formulation, reproducibility, physiological effects, and performance across procedures with different levels of invasiveness.

Several limitations should be considered when interpreting these findings. The study included only 11 animals and a single terrestrial gastropod species, which limits the precision and generalizability of the results. Only one hydrogel formulation, isoflurane concentration, and 5-min exposure period were evaluated, so dose–response and formulation–response relationships remain unknown. Isoflurane concentrations were not measured in the formulation, mucus, hemolymph, or tissues, and physiological variables were not monitored. Consequently, the mechanisms underlying topical uptake and the subsequent induction and recovery responses cannot be established from the present data. The functional scoring framework was developed to characterize behavioral and functional responses but has not been formally validated as a measure of anesthetic depth. In addition, the study did not directly assess analgesia or establish a validated nociceptive endpoint. Loss of movement or withdrawal responsiveness should therefore not be interpreted as evidence of analgesia or surgical anesthesia. Future studies should examine different isoflurane concentrations, hydrogel formulations, and exposure durations and should incorporate pharmacokinetic and physiological measurements. Validation of the functional endpoints, including site-specific reflex assessment and comparison with independent physiological or pharmacological measures, would also strengthen interpretation of anesthetic responses. Larger studies involving additional terrestrial gastropod species will be needed to assess the reproducibility and generalizability of the approach.

Conclusion

In conclusion, the present pilot study shows that an isoflurane-containing hydrogel can produce measurable and reversible changes in behavioral and functional responses in *A. fulica* under the conditions tested. The stronger response observed with Gel-Isoflurane compared with the control conditions supports further investigation of topical hydrogel delivery as a potential approach for terrestrial gastropod anesthesia. These findings should be regarded as evidence of feasibility rather than validation of a standardized anesthetic protocol or anesthetic-depth scale.

Methods

Ethical approval and study setting

This study was conducted at the Veterinary Teaching Hospital of Shahid Bahonar University of Kerman, Iran, between April and May 2026. The experimental protocol was reviewed and approved by the Biomedical Research Ethics Committee of Shahid Bahonar University of Kerman (approval No. IR.UK.VETMED.REC 2026-012). All procedures were performed in accordance with the approved protocol and with consideration of animal welfare and minimization of unnecessary handling and stimulation.

Sample size and pilot-study rationale

A formal a priori sample-size calculation was considered before study initiation. However, we could not derive an appropriate effect-size estimate because sufficiently comparable published data in giant African land snails were unavailable. In particular, no previous study was identified that evaluated a comparable hydrogel-based topical delivery system examined in the present study. The study was therefore prospectively designed as a pilot investigation, with the primary objectives of assessing the feasibility of the proposed hydrogel-based delivery approach, characterizing patterns of behavioral, motor, and reflex responses, and identifying methodological features that could inform subsequent adequately powered studies. Accordingly, the sample size was selected to provide preliminary within-animal and between-method observations rather than to provide definitive hypothesis-testing power.

Animals and husbandry

Eleven adult giant African land snails (*Achatina fulica*), approximately 1 year of age, were included in the study. At enrolment, body weight ranged from 42 to 87 g and shell length from 77 to 97 mm. Animals were housed in 40-L terraria under controlled environmental conditions, with an ambient temperature of approximately 20–25°C, relative humidity of 50–80%, and a 12-h light/12-h dark cycle. The animals had been maintained under the same husbandry conditions for at least 3 months before the experiment. Snails were provided with fresh vegetables and leafy greens as feed, and substrate was changed at regular intervals according to routine husbandry procedures. For identification, individual animals were identified by unique nail-polish markings applied to the shell.

Experimental design

We evaluated four experimental conditions in a randomized complete crossover design: Baseline observation (Baseline), 0.9% saline immersion (Immersion), gel vehicle (Gel), and isoflurane-containing gel (Gel-Isoflurane), with a 3-day washout interval between consecutive protocols. For the baseline condition, each snail was observed under its routine environmental conditions to document baseline behavioral, locomotor, withdrawal, mucus, and body tone characteristics (Fig. 3.a). For the saline immersion condition, each snail was placed in 0.9% saline (Samen®, Mashhad, Iran) for 5 minutes, with sufficient volume to immerse the entire muscular foot up to the shell opening while keeping the shell and pneumostome above the liquid level, as recommended previously for terrestrial gastropods [5, 36, 62]. For the gel vehicle condition, 2 mL of alcohol-free ultrasound gel (Hepsun®, Karaj, Iran) was applied uniformly to the dorsal surface of each snail and maintained in contact with the animal for 5 min. The gel was then completely removed using moist gauze. For the isoflurane-containing gel condition, 2 mL of

freshly prepared formulation containing corresponding to 5% v/v isoflurane (Piramal®, Mumbai, India) in the final 2-mL formulation was mixed thoroughly in a microcentrifuge tube by repeated shaking until a visually homogeneous formulation was obtained and then applied uniformly to the dorsal surface of each snail and maintained in contact with the animal for 5 min before removal (Fig. 3.b). The saline and gel conditions were included to characterize responses associated with immersion, handling, and the gel vehicle independently of isoflurane exposure. The topical procedure was adapted from previously described viscous-carrier approaches for topical isoflurane administration in amphibians [44–46].

During the experimental sessions, ambient temperature ranged from 21.1 to 24.6°C, and relative humidity ranged from 55 to 68%. The temperature of the 0.9% saline used during immersion ranged from 22 to 26°C. Behavioral and functional responses were formally assessed at 5, 10, 20, 30, and 45 min from the start of the experimental exposure. Animals were monitored throughout the observation period, but direct handling and stimulation were limited to the predefined assessment procedures.

Behavioral and functional outcome assessment

A preliminary, species-specific functional assessment framework was developed to characterize changes in behavioral responsiveness, locomotor activity, withdrawal responsiveness, mucus secretion, and body tone and posture during the experimental period (Table 4). The framework was informed by previous gastropod studies [5, 7, 8, 31, 35, 37, 52] and was designed as an exploratory functional assessment tool rather than a validated anesthetic-depth scale. Each domain was scored independently using predefined ordinal criteria ranging from 0 to 3, with higher scores indicating greater suppression or alteration of the assessed functional response. Domain-specific scores were considered the primary descriptive outcomes. An exploratory composite score was calculated as the sum of the five domain scores (range, 0–15) to provide an integrated summary of the overall response pattern. Because the individual domains may reflect distinct functional processes and the framework has not been formally validated, the composite score was interpreted as an exploratory summary measure rather than a direct measure of anesthetic depth.

This framework emphasizes functional differentiation of responsiveness rather than categorical classification of anesthesia, ensuring methodological rigor in a non-vertebrate model. This design was intended to facilitate differentiation between responses associated with the gel vehicle or immersion procedure and those observed following isoflurane exposure and to provide preliminary methodological information for subsequent studies. The same scoring structure is intended for use across future investigations, allowing direct comparison between carrier-only and drug-carrier conditions. This design was intended to facilitate differentiation between responses associated with the gel vehicle or immersion procedure and those observed following isoflurane exposure.

Recovery was assessed only following the isoflurane-containing gel condition in animals that demonstrated a clear anesthetic response. Recovery was defined as the return of all assessed behavioral, locomotor, withdrawal, mucus, and body-tone/postural parameters to their respective pre-

exposure baseline values. Recovery time was measured in minutes from removal of the isoflurane-containing gel until all assessed parameters had returned to their predefined baseline values.

Table 4
Exploratory scoring framework for assessment of functional responses in *Achatina fulica*.

Domain	Score	Description
Behavioral responsiveness	0	Active movement before stimulation (natural exploratory behavior)
	1	Discernible response, dependent on the stimulation site
	2	Irregular response, independent of the stimulation site
	3	No detectable behavioral response
Locomotor activity	0	Normal muscular leg movement (active crawling)
	1	Reduced range or continuity of movement (slow movement)
	2	Minimal discontinuous, irregular, and non-purposeful movement
	3	No active movement
Withdrawal responsiveness (<i>Tentacular withdrawal reflex</i>)	0	Complete and immediate contraction (less than 1 second)
	1	Slow and delayed contraction (more than 1 second)
	2	Very weak, partial and incomplete contraction
	3	No detectable response
Mucus secretion	0	Normal clear mucus
	1	Slight increase or decrease in mucus
	2	Abnormally copious or frothy discharge
	3	Marked dryness or complete inhibition
Body tone and posture	0	Active, extended posture (normal adhesion)
	1	Mildly decreased tone (poor adhesion)
	2	Reactive extension or contraction
	3	Stable, non-reactive extension or contraction
Composite score	0–15	Sum of individual domain scores

Footnote: Scores ranged from 0 to 3 for each domain, with higher scores indicating greater suppression or alteration of the assessed functional response. The composite score was calculated as the sum of the five domain scores (range, 0–15). The framework was developed as an exploratory functional assessment tool and was not intended to represent a validated measure of anesthetic depth.

Observer training, blinding, and consensus scoring

Four independent veterinary observers (H.G., S.S., R.L., and M.R.) participated in the behavioral assessments. Before formal data collection, observers were trained using the predefined scoring rubric and operational definitions for each behavioral domain. At each predefined observation time point, all four observers independently evaluated each animal and assigned scores for all behavioral domains. Whenever feasible, observers were blinded to treatment identity and experimental sequence during scoring. To ensure consistency, operational definitions were reviewed before each scoring session, and ambiguous responses were recorded conservatively.

For each animal, experimental condition, observation time point, and behavioral domain, the final consensus score was defined as the median of the four independent observer ratings. The resulting median-based consensus dataset was used for all subsequent analyses.

Statistical Analysis

All statistical analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria). Interobserver reliability of individual behavioral domains was assessed using Fleiss' κ and Krippendorff's α . Reliability of the composite score was evaluated using the single-measure consistency intraclass correlation coefficient (ICC3,1) with 95% confidence intervals. Pairwise agreement between observers was assessed using quadratic weighted Cohen's kappa. Agreement was interpreted according to the criteria proposed by Landis and Koch [63], where κ values of 0.61–0.80 indicate substantial agreement and values > 0.80 indicate almost perfect agreement.

For all subsequent analyses, a consensus dataset was generated by calculating the median score of the four independent observers for each behavioral domain, animal, experimental condition, and observation time point. This consensus dataset was used for all descriptive and inferential statistical analyses. Descriptive statistics are presented as median and interquartile range (IQR) for each behavioral domain and composite score.

Two complementary non-parametric repeated-measures analyses were performed. First, behavioral responses at the 5-minute observation were compared among the four experimental conditions (Baseline, Immersion, Gel, and Gel-Isoflurane) using the Friedman test for repeated measures, with individual animals treated as matched subjects. When the Friedman test was significant, pairwise Wilcoxon signed-rank tests with Holm correction for multiple comparisons were performed as post hoc analyses. Second, the temporal progression of anesthesia within the Gel-Isoflurane treatment was evaluated using the Friedman test across the five observation time points (5, 10, 20, 30, and 45 minutes). Significant overall effects were followed by paired Wilcoxon signed-rank tests with Holm-adjusted P values.

For all Friedman analyses, Kendall's coefficient of concordance (Kendall's W) was calculated as a measure of effect size and interpreted as negligible (< 0.10), small (0.10–0.29), moderate (0.30–0.49), or

large (≥ 0.50).

All statistical tests were two-sided, and a P value < 0.05 was considered statistically significant.

Declarations

Competing interests

The authors declare no competing interests.

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Author Contribution

Conceptualization, R.L., H.G., S.S., and M.R.; methodology, R.L., H.G., S.S., and M.R.; software, R.L., and H.G.; validation, R.L., H.G., S.S., and M.R.; formal analysis, R.L., and H.G.; investigation, R.L., H.G., S.S., and M.R.; resources, R.L., H.G., S.S., and M.R.; data curation, R.L., H.G., S.S., and M.R.; writing—original draft preparation, H.G.; writing—review and editing, R.L., H.G., S.S., and M.R.; visualization, R.L., H.G., S.S., and M.R.; supervision, H.G., and S.S.; project administration, H.G., and S.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The datasets generated during and/or analyzed during the current study are available in the Zenodo repository, <https://zenodo.org/records/22925416?>

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Figures

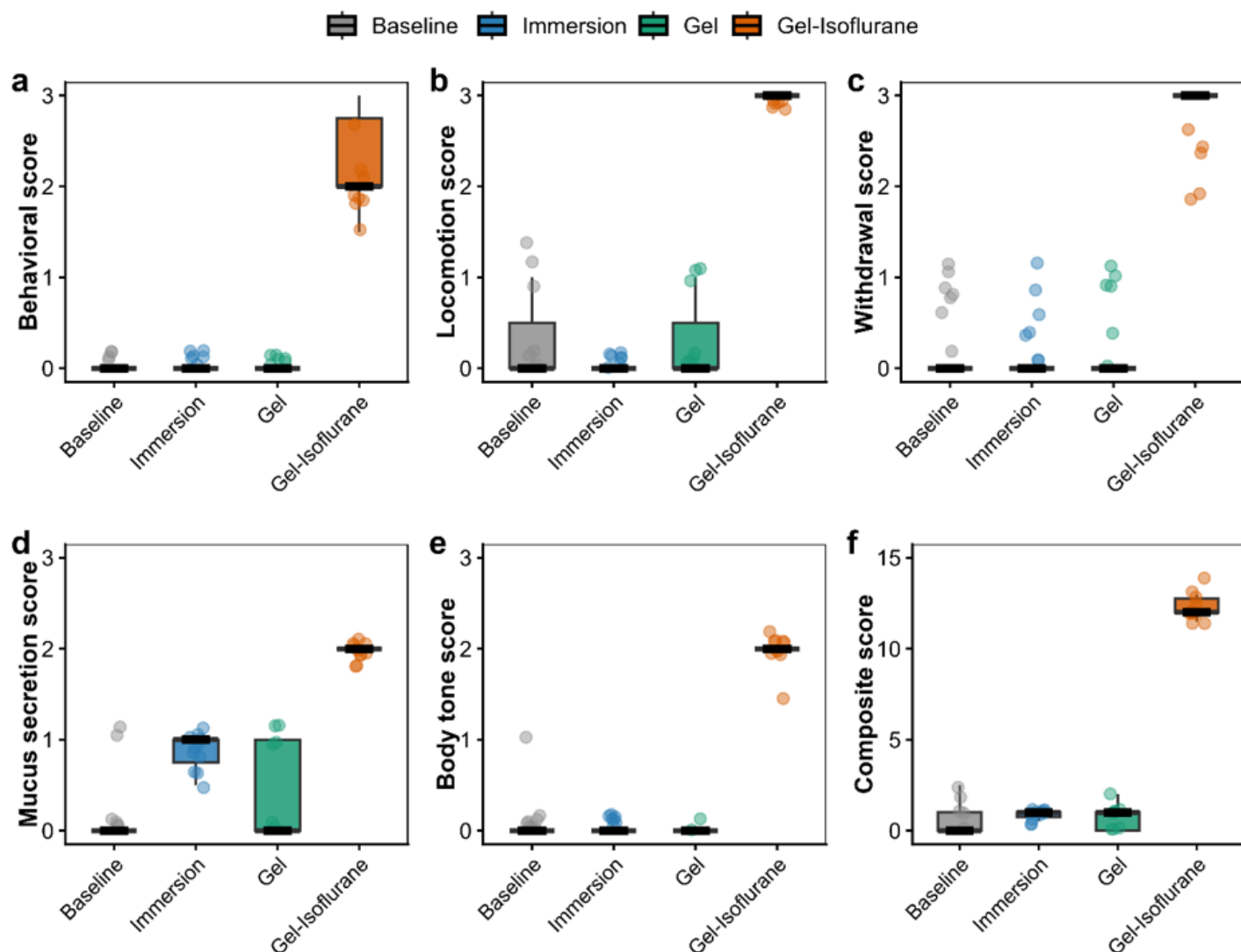


Figure 1

Consensus scores across experimental conditions at 5 min after treatment. Boxplots show the median (horizontal line), interquartile range (box), and $1.5 \times$ interquartile range (whiskers); individual dots represent individual animals ($n = 11$ per condition). Consensus scores were calculated as the median of four independent observer ratings. (a) Behavioral score, (b) Locomotion score, (c) Withdrawal score, (d) Mucus secretion score, (e) Body tone score, and (f) Composite score.

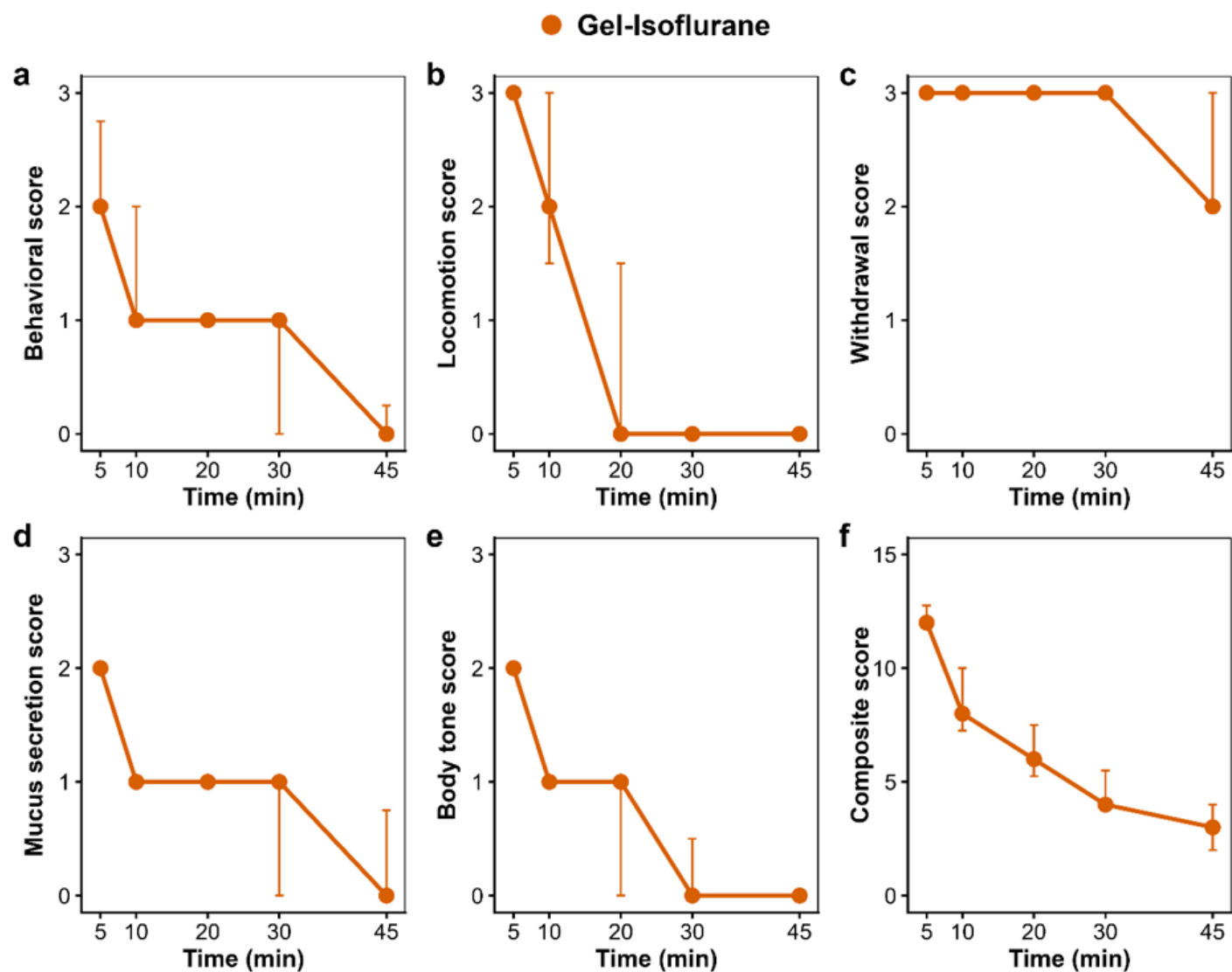


Figure 2

Temporal changes in consensus scores following Gel-Isoflurane exposure. Points represent median consensus scores and error bars indicate the interquartile range (IQR; $n = 11$). Consensus scores were calculated as the median of four independent observer ratings at each observation time. The hydrogel was removed at 5 min. Time points indicate minutes after initiation of Gel-Isoflurane exposure. (a) Behavioral score, (b) Locomotion score, (c) Withdrawal score, (d) Mucus secretion score, (e) Body tone score, and (f) Composite score.

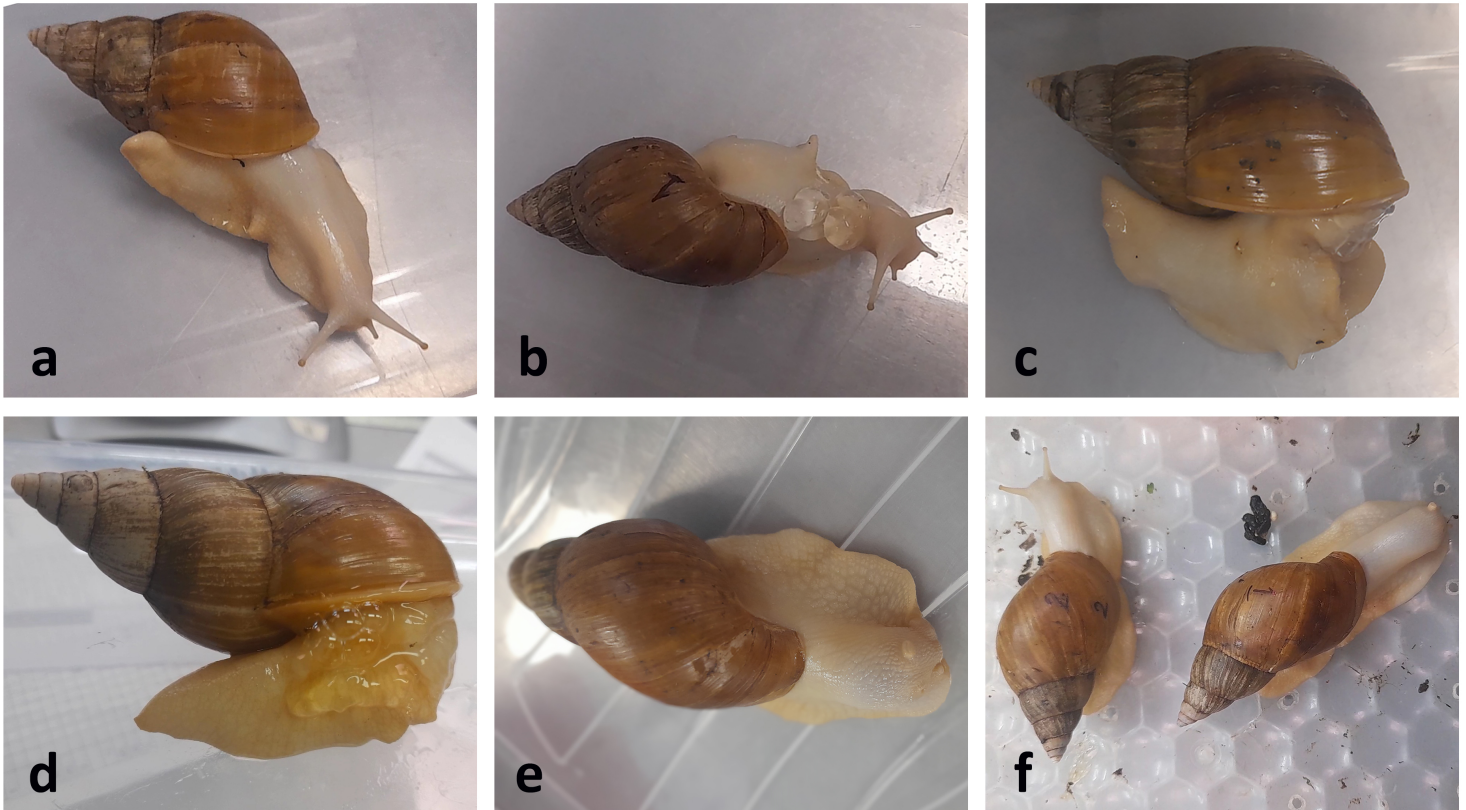


Figure 3

Representative behavioral and functional observations during Gel-Isoflurane exposure and recovery.

Representative photographs illustrating selected responses observed during topical isoflurane exposure and subsequent recovery in *Achatina fulica*, including **(a)** active movement before gel exposure, representing natural exploratory behavior; **(b)** topical application of the gel to the dorsal surface of the snail; **(c)** marked reduction in locomotor activity, contraction, and altered body posture during the anesthetic response; **(d)** abundant foamy mucus secretion during the anesthetic response; **(e)** return of an active extended posture and normal locomotion while tentacular withdrawal remained absent during the recovery phase; and **(f)** return to the predefined baseline functional state (left) alongside persistence of absent tentacular withdrawal during the recovery phase (right). These photographs are provided as representative qualitative observations and are not intended as independent quantitative measures of anesthetic depth.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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