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Research Article

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Calamus oil as an anesthetic for *Cyprinus carpio* (Ornamental Koi)

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ABSTRACT

The anesthetic effect of Acorus calamus oil in fish is reported for the first time. The anesthetic time was evaluated in terms of the time taken from induction to recovery. Through a laboratory experimental design the Koi carp (37.6 ± 4.27 g) was short bath treated in water containing calamus oil (dissolved in methanol) at different concentrations (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mg L^{-1}). The time taken for induction of anesthesia is negatively related to concentration of the calamus oil used while the recovery time had a direct relationship. Based on the opercular activity, fading of ventilation and recovery time 2.5 mg L^{-1} of calamus oil has been found to be ($P<0.05$) ideal. At this concentration anesthesia was induced within 8.03 ± 0.01 minutes; the fish remained amenable for easy handling without any reflex action for 8.05 ± 0.02 minutes and all the exposed fishes in the anesthetic solution recovered 43.02 ± 0.02 minutes. The results demonstrate that this phyto anaesthetic is effective for sedation and anesthesia of Cyprinus carpio. A concentration of 2.5 mg L^{-1} calamus oil showed rapid anaesthetic and recovery times in the C. carpio, indicating its suitability to minimize the handling stress during weighing, measuring, tag implantation and for biopsy assay. This ideal dosage did not make any physiological changes in the treated fishes.

Keywords: *Phytoanesthetic, Acorus calamus, Cyprinus carpio, Koi, Anesthetic.*

INTRODUCTION

Anesthetics are important in fish culture to minimize handling stress and mortality. A number of chemicals are used as fish anesthetics, but the most commonly used anesthetics, e.g. tricaine methanesulphonate (MS-222), quinaldine and 2-phenoxyethanol, are toxic and expensive[1,2]. In many countries the use of fish anesthetics is a matter of concern for the consumer since there are no specific laws regulating their use. Usually the recommendations of the US Food and Drug Administration (FDA) are followed. Acquisition of MS-222 is also difficult, it is not locally produced and if the fish is meant for export it should obligatorily undergo a 21-days withdrawal period[3]. Traditional chemicals such as urethane, ether and chloroform used to anaesthetize fish are now restricted because they all contain carcinogens[4]. Clove oil and carbon dioxide are less harmful chemicals to the researcher, the latter being known as a fish anaesthetic for over 50 years[5]. Carbon dioxide, however, is considered as only partially effective, and is slow in action and lethal after repeated exposures[6]. They have been used for centuries as a topical anaesthetic in Indonesia[7], and local anaesthetic in dentistry[8]. In aquaculture, it has been widely used to anaesthetize freshwater and marine fishes and molluscs[9,10,5,11].

However, unlike MS-222, lidocaine hydrochloride and clove oil do not require a withdrawal period since they do not contain environmentally harmful elements; therefore they are considered more appropriate for

use in aquaculture. Besides MS-222 is expensive and costs 10 times more than a similar dose of benzocaine[12,]. Therefore, it is necessary to evaluate local safe alternatives as fish anesthetic.

Acorus calamus is a traditional plant of Indian Ayurvedic medicine, containing a group of phytochemical compound that inhibits the *in vitro* growth of gram–negative bacteria *Aeromonas hydrophila*[13], and in vivo short bath treatment of *Cyprinus carpio* experimentally infected with *A. hydrophila*; while fixing the duration of exposure we found that the fish were apparently sedated when exposed for more than 10 minutes (<2 mg/l). Hence this study was conducted to find out the possibility of using this as an anaesthetic for fishes choosing *Cyprinus carpio* as animal model.

MATERIAL AND METHODS

Plant material and extraction

Acorus calamus Linn commonly known as sweet flag is an aromatic medicinal plant belonging to the Araceae family. The fresh rhizome *A. calamus* collected from Bharathidasan University campus, Trichirappalli, Tamil Nadu, India were washed under running tap water; after removing the small hairs the rhizomes were finely chopped, shade dried to attain weight consistency, then homogenized to fine powder in an electric blender and stored in airtight bottles. For organic solvent completely dried powder of plants extracted with 90% w/w ethanol using a soxhlet apparatuses. The ethanol was removed under pressure using a rotary evaporator. The dried residue crude extracts were stored in a dark bottle at 4 ºC in air tight bottles for further studies[14].

Isolation of components

The active ethanol residue of *A. calamus* was submitted to chromatography over on silica gel (32 g) eluted with a gradient system of increasing polarity (hexane, dichloromethane, ethyl acetate and methanol). Such as ethyl acetate 20% in Hexane (20:30) - (8.1 mg), the fractions F_2 assed against *A. hydrophila.*

Chemical analysis of essential oils

The crude extract of essential oils was washed with NaCl solution, dried on sodium sulfate and evaporated under vacuum in a rotary evaporator. Gas chromatography coupled with mass spectrometry was used to identify the main volatiles released by each essential oil. GC-MS analysis was performed using a Perkin-Elmer Turbomass system with a split-split less PSS injector and a fused-silica capillary column (30 m by 0.32 mm) with a thick methylsilicone coating (4 m). The carrier gas was 99.99% helium at 1.5 ml/min for the 10-m column length. The column temperature program was 5 ºC/min, from 70 to 250 ºC. Total ion chromatograms and mass spectra were recorded in the electron impact ionization mode at 70 eV. The transfer line and the source temperature were maintained at 150 ºC.

Isolation of purified essential oil

The rhizome of *A. calamus* were stream distillation of rhizomes gave calamus oil (1.7% w/w), which after column chromatography on a silica gel column with hexane / ethyl acetate (99:1 to 90:10) provided 1 (82% w/w) as pale yellow liquid (R_f 0.39 on silica gel TLC plate in 4% ethyl acetate in hexane) and its spectral data agreed well with reported literature value.

Fish (*C. carpio*)

The Koi carp *C. carpio* (37.6 ± 4.27 g and length 26.72 ± 0.77 cm) were used as the experimental animal in this study. The fish were acclimatized in tanks (2000 L capacity; 6×4×4 m) to the laboratory condition for at least 7 days. They were not fed for 48 hrs period before the commencement of the experiment. The physicochemical conditions recorded are: Temperature 28 ± 2 ºC, dissolved Oxygen 3.4 ml/l, total alkalinity 52 ppm, total hardness 14.0 N, pH = 7.0.

Experimental anesthetic

The stock solution of calamus oil was dissolved in methanol at a ratio of 1:10[15] and then diluted in water to produce different concentrations of anesthetic. Groups of ten fish were placed in a 10 L transparent plastic tank containing 5 L water treated with different concentrations of anesthetic. After quantifying the anesthetic time for each individual, that animal was immediately transferred to a recovery aquarium with 5 L of well-aerated water under a controlled temperature.

Experimental design

Experiment 1: Determining the lowest effective concentration of calamus oil

The fish were individually placed in the test aquarium (capacity 700 L) containing water mixed with one of the required concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg L^{-1} of calamus oil) and held there until the fish did not evince any reflex action to handling. To assess the depth of anesthesia further the fish were removed from the water in a hand net. If there was a response (jerking movement), the exposure was continued; if there was no reaction, the fish were transferred to the recovery tank containing plain water and observed until they recovered and the normal activity was restored. The time of induction and recovery was measured from the time of introduction of the fish into the recovery tank (in minutes) and changes in the behavior during recovery were noted[3], to fix the ideal dose of the anaesthetic required.

Experiment 2: Relationship between the exposure time and recovery behavior at 2.5 mg/l concentration.

Fish were individually exposed to calamus oil solution for 10, 20 and 30 min, and then removed on induction of anesthesia. The fish were then placed in recovery tanks containing well aerated fresh water and their recovery was noted for a period of 96 hrs.

Experiment 3: Effect of anesthetic on subcutaneous needle puncture in Koi carp

On the ideal dose exposure time of anesthetic was established in experiment 1 and 2 was evaluated on Koi carp. Anesthesia by simulating a bleeding event using a subcutaneous needle puncture and recording the presence or absence of a reflexive response. 2.5 mg·L^{-1} were added to 1 L of water and fish were anaesthetized as described above. Once fish reached stage II, a hypodermic needle (2-8 G) attached to a tuberculin syringe was inserted into the caudal vasculature and the presence or absence of a reflexive response was recorded (Table 3).

Blood sampling and Laboratory assays

Blood samples from the caudal severance. While anesthetic the substance can elicit a cortisol response[16], exposure to level > 100 mg/l does not produced immediate changes in serum cortisol[17], that could confound experimental result. Haematocrit values were determined and blood smears made for differential leucocyte (while blood cell, WBC) counts. The remaining blood was allowed to clot at about 4 ºC and centrifuged to collect serum. The serum was stored at -80 ˚C for later analysis of cortisol, glucose concentration and lysozyme activity. Serum cortisol was determined with iodine -125 (I 125) radioimmunoassay kit serum glucose was measured with an enzyme - based colorimetric diagnostic kit (sigma). Serum lysozyme activity was determined by the lysoplate method with sodium phosphate buffer at pH 6.24 value[15]. Different leucocyte counts were obtained from blood smears fixed in methanol, stained in a Wright - Giemsa combination stain solution and examined under microscopy. The results are presented as total leucocyte abundance.

Histological assay

The tissue samples of fish were fixed with 10 % neutral buffered formalin for 24 hrs, Fixed tissues were processed routinely, Embedding in paraffin wax sectioning and stained (following standard histological procedure for fish - Roberts 1989), Section were cutting at 5 um thickness, Stained with haematoxylin and Eosion (Qualigens, Mumbai), Mounted with DPX mountant, Mounted slides were viewed using OLYMPUS Microscope at 40X Magnification.

RESULTS AND DISCUSSION

Experiment 1:

The *A. calamus* oil administered at the concentrations ranging from 1 to 5 mg·L^{-1} resulted in progressive anesthesia. After induction of anesthesia the fish on transfer to a tank with clean water recovered. The symptoms of anesthesia[3], faded in reverse order. The mean times of the duration of anesthesia and the recovery symptoms are presented in Table 2. The time for taken for sedation is directly related to the concentration of the calamus oil used and shortening of the induction time of anesthesia; a concentration of 2.5 mg L-1 resulted in sedation only while higher concentrations of 4.0, 4.5, 5.0 mg L^{-1} led to the equilibrium disturbances in all fish.

Experiment 2:

C. carpio on exposure to 2.5 mg L^{-1} reached Stage II (Table 1) in 1.25 ± 0.30 min. Doses lower than 2.0 mg L^{-1} resulted in longer induction times (Table 2). Fish exposed to 5 mg L^{-1} of *A. calamus* oil reached Stage II in just seconds. While those tested at 10 mg L^{-1} did not reach Stage II within the pre-determined less than 4 seconds exposure period. Fish at 8 or 9 mg L^{-1} reached stage II within 9 seconds respectively, while Fish in 7 mg L^{-1} failed to reach stage II within the 20 seconds period. Recovery to stage VI occurred in 43 min for 2.5 mg L^{-1} anesthetics tested.

Experiment 3:

A single ideal concentration of anaesthetic was tested on sub adults for its ability to prevent a reflex reaction to a subcutaneous needle puncture. All of the fish anaesthetized in essential oil (2.5 mg L^{-1}) and reacted to the needle puncture (Table 3).

Short term–recovery physiological responses

Haematocrit levels, serum cortisol, serum glucose, serum lysozyme activity, differential leucocyte counts were not significantly different from the control group (Table 4).

Histological analysis

Ceratobranchial bone of the gill arch (Figure A), sagittal section showed 1. acellular zone; 2. hyphertrophic zone; 3. growth zone; 4. apical zone; 5. abductor muscle; 6. efferent branchial artery; 7. mucosal epithelium; 8. primary lamella and 9. secondary lamellae. Pseudobranch, sagittal section showed (Fig. B), 1. afferent psedobranchial artery containing red blood cells; 2. secondary psedobranchial lamella; 3. glandular psedobranch cell and 4. epithelial cell. Gill filament, sagittal section through cartilaginous support showed (Fig. C), 1. primary lamella; 2. extracellular cartilaginous matrix; 3. chondrocytes; 4. secondary lamella; 5. epithelial cells; 6. mucous cell; 7. pillar cell; 8. lacuna (capillary lumen); 9. red blood cells within lacuna. They were indicating no cytological difference occurred in the anesthetic fish.

This work is demonstrated calamus oil to be a suitable anaesthetic for aquaculture and fisheries use anesthesia is a biological state induced by an external agent, which results in the partial or complete loss of sensation or loss of voluntary neuromotor control through chemical and non chemical means[3]. In fisheries research and aquaculture operations, anesthetics are necessary to minimize stress and physical injury during various handling procedures (e.g. weighing and measuring, tagging, sampling). The choice of anaesthetic generally depends on several considerations: (1) availability, (2) cost – effectiveness (3) ease of use (4) nature of the study (5) safety to the user. Criteria that determine the efficacy of an anaesthetic include; 1. Quick Induction of anesthesia allowing handling of fish, 2. Full recovery as assessed by normal swimming activity and 3. Absence of any mortality, after 15 minutes of recovery[18-20]. Under field conditions an anaesthetic also should: (1) have swift induction of, and recovery from, anesthesia; (2) not excessively disturb the physiological balance of the fish, reducing its chances of survival upon release and (3) allow for the immediate release of the fish into the food chain, minimizing effects on ecological processes[21-23], it also should not have any potential side-effects on fish, humans and the environment[6]. Beta asarone of the calamus oil may fulfill the above needs and can be additional safe anaesthetic for aquaculture studies and field studies such as tagging.

The chemicals that have historically been used for anesthesia of fish were originally developed for other purposes. As a result, the potential side-effects of these chemicals on humans were not investigated thoroughly[24]. Quinaldine (2-methylquinoline) has been widely used in fisheries research but has recently been associated with thyroid abnormalities in humans and mice. In recent years, biologists and aquaculturists alike have been searching for alternative anesthetics that give the required results, and are safe for humans.

This study indicates that the *A. calamus* oil can be a safe alternative fish anaesthetic for *C. carpio*. Beta asarone is the active compound of essential oil, is obtained from the rhizome of *A. calamus*. It has several advantages in fisheries research, assessment studies and aquaculture application. It is an easily and inexpensively obtained organic distillate that is used as a food additive and possesses antifungal and antibacterial properties[25].

Its potency to ward off the *in vitro* growth of fish pathogen *A. hydrophila* has been documented[13]. Because it is an organic compound withdrawal period may not be required for fish intended for human consumption. In addition, it may not pose a chemical health hazard to the consumer since this has been a traditionally used home remedy for a long time. A standard dose of 2.5 mg L^{-1} is ideal for the sub-adult *C. carpio*; this dosage can be suitably modified for a range of fish species. Since calamus oil is shown to be an efficacious and safe anaesthetic for *C. carpio.*

Anesthetization in calamus oil does not appear to alter the physical artifacts in blood samples collected at stage 5 inductions and recovery (Table 4). Chemical anesthetics result in variations in physiological profile[26-28], but calamus oil does not cause any significant variations on haematocrite, serum cortisol, glucose level, serum lysozyme activity and increased in serum glucose levels at post recovery when compared to the control. Small 2003 found that plasma cortisol levels remained at baseline levels with clove oil during 30 minutes of anapestic in the channel cat fish *Ictalurus punctatus,* while MS-222 anesthetized catfish showed an eight – fold increase in cortisol levels over the same period. Blood plasma chemistry[29], found significant reductions in the amount of absolute fatty acid levels in response to MS-222 to the point of respiratory failure when compared to control group which were not anaesthetized. However, total protein and sodium levels remained unchanged, for the reported[30]. This considerable and extended increase in serum lysozyme activity in anesthetized fish as found in this study has also been reported[16,34-36]. Thus this is concluded that induced mortality is not occurred at the ideal dosage; further no adverse cytological changes occurred in the anaesthetized fish.

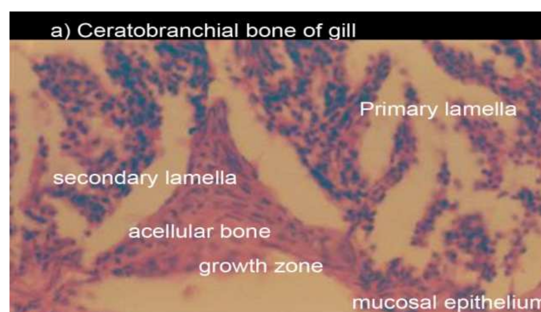

Figure A. Ceratobranchial bone of the gill arch, sagittal section

1. acellular zone; 2. hyphertrophic zone; 3. growth zone; 4. apical zone; 5. abductor muscle; 6. efferent branchial artery; 7. mucosal epithelium; 8. primary lamella and 9. secondary lamellae.

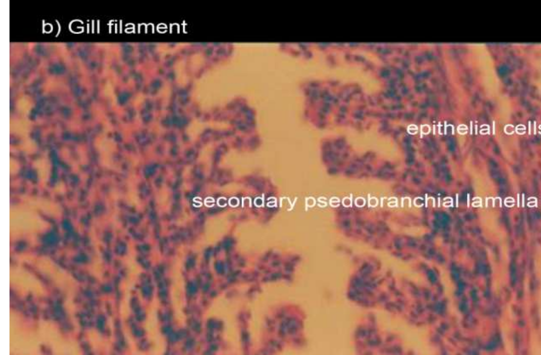

Figure B. Pseudobranch, sagittal section

1. afferent psedobranchial artery containing red blood cells; 2. secondary psedobranchial lamella; 3. glandular psedobranch cell; 4. epithelial cell.

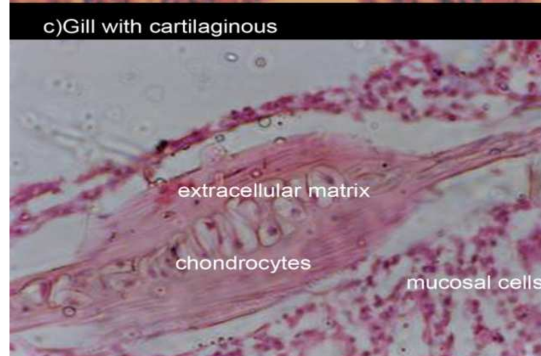

Figure C. Gill filament, sagittal section through cartilaginous support

1. primary lamella; 2. extracellular cartilaginous matrix; 3. chondrocytes; 4. secondary lamella; 5. epithelial cells; 6. mucous cell; 7. pillar cell; 8. lacuna (capillary lumen); 9. red blood cells within lacuna.

Table 1: *C. carpio*: Duration (mean ± SD min) of anesthetic events ♣ on exposure to various concentrations of calamus oil Beta asarone

Conc (mg/ml)	Loss of reactive to stumuli	Loss of equilibrium	total loss of equilibrium	loss of reflex reactivity	Reduced opercular movement	Cessation of opercular movement
1.0	0.41±0.11	0.92±0.37	0.72±0.47	2.81±0.57	3.71±0.67	15.52±2.37
1.5	0.34 ±0.01	0.83±0.18	1.64±0.66	2.67±0.82	3.43±0.95	12.58±3.53
2.0	0.30 ±0.02	0.63±0.01	1.42±0.18	2.07±0.03	2.42±0.13	09.62±4.16
2.5	0.25 ±0.09	0.43±0.46	1.22±0.18	1.42±0.44	1.71±0.50	07.63±0.04
3.0	0.19 ±0.05	0.31±0.26	0.82±0.92	1.20±0.12	1.62±0.03	01.05±2.01
3.5	0.15±0.03	0.22±0.21	0.74±0.21	1.15±0.04	1.57±0.01	00.32±5.71
4.0	0.10±1.02	0.17±0.40	0.66±0.23	0.98±1.20	1.43±0.23	00.04±0.41
4.5	0.80±2.40	0.12±4.51	0.52±0.51	0.84±1.03	1.36±2.04	00.03±0.45
5.0	0.50±0.13	0.91±3.02	0.32±4.12	0.62±0.02	0.85±0.13	00.02±0.04

♣ Events as described by Summerfelt and Smith, 1990

Table 2: *C. carpio*: Duration (mean ±SD min) of recovery events ♣ (Summerfelt, 1990) on exposure to various concentrations of calamus oil Beta asarone

Concentration	Reappearance of opercular movement	Partial recovery of equilibrium	Total recovery of equilibrium	Stolid response to external stimuli	Normal swimming
1.0	20.01 ± 0.02	21.23 ± 0.12	22.04 ± 0.23	24.03 ± 3.21	28.01± 0.23
1.5	25.08 ± 0.13	27.01 ± 2.31	28.16 ± 0.21	29.15 ± 0.23	29.57±0.01
2.0	29.32 ± 1.23	31.01 ± 0.09	33.02 ± 0.32	34.02 ± 0.12	37.05± 0.12
2.5	35.02 ± 0.91	38.03 ± 1.20	39.51 ± 0.73	41.23 ± 0.13	43.02±0.02
3.0	41.11 ± 0.23	43.03 ± 0.23	45.31 ± 0.23	46.34 ± 0.19	49.14±3.41*
3.5	47.04 ± 1.02	48.13 ± 0.15	50.31± 0.12	53.01 ±2.03	55.20 ± 0.21*
4.0	59.21 ± 2.60	62.01 ± 2.10	65.02 ± 0.19	68.06 ± 0.14	70.24 ± 1.05*
4.5	63.04 ± 3.18	65.01 ± 0.20	68.22 ± 2.01	72.42 ± 0.04	82.42 ± 0.12*
5.0	68.30 ± 0.15	72.04 ± 2.3	78.03 ± 0.21	89.03 ± 3.02	105.03 ± 0.34*

* Staggered movement occurred; ♣ Events as described by Summerfelt and Smith, 1990

Table 3: *C. carpio*: Duration (minutes) of exposure and behavior events during anesthetic time from the time of induction

Duration	Events ♣
00.00 ± 0.00	Induction
00.25 ± 0.09	Loss of reactive to stimuli
00.43 ± 0.46	Loss of equilibrium
01.22 ± 0.18	Total loss of equilibrium
01.42 ± 0.44	Loss of reflex reactivity
01.71± 0.50	Reduced opercular movement
07.63 ± 0.40	Minimal opercular movement
08.01 ± 0.01	Fading ventilation
08.03 ± 0.01	Deep sedation
08.05 ± 0.02	Loss of reflex activity
43.02 ± 0.02	Recovery

♣ Events and the corresponding stages as described by Summerfelt and Smith, 1990.

Table 4: Changes in physiological parameters (mean ± SE minutes) in *C. carpio* before (Preanaesthesia), deep sedation (5th stage) after induction and recovery

Parameter	Preanaesthesia	Induction (5th stage)	Recovery
Haematocrit	20.9 ± 0.03	21.01 ± 0.02	20.7 ± 1.21
Serum cortisol(ng mL^{-1})	48.3 ± 0.12	62.2 ± 1.21	57. 0 ± 0.32
Serum glucose (mg dl^{-1})	40.4 ± 0.31	32.23 ± 0.24	38.12 ± 0.10
Serum lysozyme activity(units mL^{-1})	41202.0 ± 0.23	39301.2 ± 2.1	3017.2 ± 2.3
Total leucocyte (%)	2.82 ± 2.41	2.76 ± 2.30	2.84 ± 0.21
Neutrophile (%)	8.71 ± 0.63	8.62 ± 3.12	8.69 ± 1.01
Eosinophile	0.42 ± 0.03	0.45 ± 1.20	0.43 ± 0.30
Basophile	0.15 ± 0.08	0.10 ±2.07	0.12 ± 0.21
Monocytes	7.60 ±0.40	7.15 ±1.72	7.52 ±0.42

Stages of Anesthesia	Description
I	Loss of equilibrium
II	Loss of gross body movements but with continued opercular movements
III	As in Stage II with cessation of opercular movements
Stages of Recovery	**Description**
I	Body immobilized but opercular movements just starting
II	Regular opercular movements and gross body movements beginning
III	Equilibrium regained and preanesthetic appearance

From, Iwama et al., 1989

CONCLUSION

Calamus oil is a good alternative as a fish anaesthetic. It is relatively inexpensive and is generally regarded as safe for the user and for the fish. The fish were sufficiently sedated for normal sampling (length, weight, scale sample) in slightly over one minute, with an anaesthetic concentration. It is an inexpensive and effective anaesthetic. It should not require a withdrawal period, since it has an accepted human daily intake. It would be valuable to have studies conducted under controlled conditions to determine the anti fungal and anti bacterial properties of calamus oil on fish.

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