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Cover Photograph: A challenger *Betta* fish (left) faces a Crown Tail *Betta* fish (right) in an encounter trial. Both animals are exhibiting frontal display behavior, wherein fish face their opponent and flare operculum.. Photograph © Holly Boettger-Tong and Stephanie DeMer.

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Lavender Essential Oil Reduces Aggression in Male *Betta splendens*

Ashleigh Woods[1] and Holly Boettger-Tong[2*]

Abstract - Lavender essential oil (LEO) is distilled from the plant *Lavender angustifolia* and is commonly used for aromatherapy in humans. In recent years, it has been used on various fish species as an anesthetic during medical procedures and as a sedative during fish transport. The effect of LEO on agonistic behaviors in fish has not been explored. This study aimed to investigate LEO's effectiveness at reducing aggression in male Crown-Tail (CT) *Betta splendens* (*Betta* fish). To determine the effect of LEO treatment on *Betta* fish agonistic behaviors, a pre/post study design was conducted. The data indicate that LEO significantly reduced aggression in male CT *Betta* fish and that this effect was partially reversible with time. A pilot dose response study with linalool, the active ingredient in LEO, was also completed to determine if this compound might be able to alter behavior. Concentrations of 15 ppm, 30 ppm, and 60 ppm were tested on male CT *Bettas*; the data indicate that agonistic behavior was significantly inhibited versus Control at 60 and 30 ppm linalool, but not at 15 ppm. The data from this study suggests that lavender essential oil, and its major constituent linalool, can be used to alter agonistic behavior in *Betta splendens*.

Introduction

Originating in Southeast Asia, *Betta splendens* Regan (*Betta* fish) are common household pets in the United States, often purchased by new fish owners due to their robustness. Wild type *Betta* fish are extremely aggressive, mostly due to their territorial nature and the obligations of resource competition. They exhibit agonistic behaviors to fight and display dominance against trespassers, which are often other *Betta* fish. Agonistic behaviors are shown in threatening situations for the organism, for example competition, defense, and submission (Ramos and Goncalves 2019). For *Betta* fish, these behaviors include charge, frontal display, caudal swing, and lateral display (Table 1).

Commercially, *Betta* fish are bred for aggression; males are often placed in matches with each other where they fight to the death, with the winner of these contests being used for breeding stock (Ramos and Goncalves 2019). Both in the wild and in households, *Betta splendens* exhibit specific agonistic behaviors in response to stress stimuli. To date, there is limited research about substances that can reduce aggression in these fish. One study investigating Prozac as an environmental pollutant (Greaney et al. 2015) described behavioral alterations in *Betta splendens* aggression, but this was not a systematic study of the efficacy of this compound in reducing fish agonistic behaviors. As *Betta splendens* are commonly transported and sold under conditions which stimulate agonistic behaviors and are physiologically stressful, identifying substances that might inhibit these behaviors without causing anesthesia could be useful in reducing stress and prolonging health in these organisms. The ornamental fish trade is a fast-growing, multibillion-dollar, world-wide industry involving over 2500 species of fish. From 2000–2014, global exports of ornamental fish rose

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from $177.7 million USD to $347.5 million USD. In the US, the market was valued at USD 5.4 billion in 2021 (Dey 2022). Transportation of these fish is physiologically stressful, leading to poor health outcomes and in some cases, is suspected to cause mortality (Souza et al. 2019). Fish are transported in sealed plastic bags filled with minimal water which causes ammonia and carbon dioxide buildup, disrupting fish homeostasis (Becker et al. 2016, Lim et al. 2003, Paterson et al. 2003). The increase in ammonia and carbon dioxide changes pH levels and ultimately induces a stress response, releasing catecholamines and corticosteroids. This causes immune, metabolic, endocrine, gene, and energetic changes; repeated or prolonged exposure to this stress decreases growth, reproduction and development and causes negative changes in behavior and disease resistance (Schreck and Tort, 2016). Treatment with a substance that can alter stress and behavior, or with a sedative administered prior to the stress exposure, can decrease the risk of these physiological consequences (Souza et al. 2019).

In recent years, many studies have tested the efficacy of essential oils as sedatives and anesthetics for a variety of fish species to determine whether these oils can be used as an alternative to chemical anesthetics, such as MS-222 (reviewed in Aydin et al. 2020). Among these essential oils is lavender essential oil (LEO). Its major components are linalool, linalyl acetate, 1,8-cineole *B*-ocimene, terpinen-4-ol, and camphor. The quantity of these ingredients is dependent upon the species of flower it is derived from. LEO derived from the flower *Lavender angustifolia* Miller is mainly composed of linalyl acetate (3,7-dimethyl-1,6-octadien-3yl acetate), linalool (3,7-dimethylocta-1,6-dien-3-ol), lavandulol, 1,8-cineole, lavandulyl acetate, and camphor (Koulivand et al. 2013). Thus LEO, as well as other essential oils, is a mixture of botanicals; however, the two main ingredients of LEO are linalool and linalyl acetate (Koulivand et al. 2013). Of the main active ingredients, linalool has been found to be responsible for an anesthetic effect on fish (Leal-Cardoso et al. 2010). These studies demonstrated linalool's ability to act on the somatic nervous system, with the authors postulating that this chemical depresses the central nervous system and ultimately the stress response.

Table 1. The aggressive behaviors observed in this study. Each time either behavior was displayed, it counted as 1 aggressive behavior. Photos are screen captures of male Crown-Tail *Betta* fish from the data analysis videos of this study. Table design is modified from Ramos et al. (2019).

Body Position	Behavior	Description
	Frontal Display	Faces the opponent and flares operculum and unpaired fins.
	Caudal Swing	The caudal fin is waved toward the opponent. Usually results in a quick turn.

Lavender essential oil is commonly used by humans for aroma and skin therapy, and there are non-peer-reviewed reports that it can reduce symptoms of anxiety, depression, and insomnia. Though there are no research studies to support these anecdotal claims in humans, LEO has been used successfully on fish, specifically blue dolphin cichlids and rainbow trout, as an anesthetic (Can and Sümer 2019, Metin et al. 2015). Other oils such as tea tree, clove eucalyptus, and mint have sedative capacities at select doses (Rezende et al. 2017). Although multiple studies have demonstrated the efficacy of lavender essential oil as an anesthetic (Metin et al. 2015, Can and Sümer 2019), this study aimed to determine if LEO can be used to reduce aggression in male Crown-Tail *Betta splendens* (CT). Lavender essential oil was tested to determine its effect on agonistic behaviors of male CT *Betta* fish in the context of repeated challenges by Veil Tail *Betta* opponents and to determine the reversibility of this effect on *Betta* behavior. A further study was conducted to determine the effect, if any, of purified linalool on *Betta* agonistic behaviors.

Methods

Animals

For the LEO experiments, six male Crown-Tail *Betta splendens* (CT) and three male Veil Tail *Betta splendens* (VT), to act as challengers, were purchased from PetSmart (Macon, GA). For the linalool experiment, a total of 32 CT *Betta splendens* were used, with four Hellboy *Betta splendens* used as challengers; all fish were obtained from LiveAquaria (Rhinelander, WI). Fish for all experiments were housed in plastic containers with approximately 700 mL of Spring water; during trials, the CT and VT were transferred to separate plastic testing containers (TC) for treatment and behavioral observations, then returned to their housing container (HC). To minimize stress outside of trials, fish were kept visually separated from each other with white paper dividers between each container. They were fed Aqueon *Betta* food daily and partial water changes were completed weekly. Water changes consisted of transferring the fish from HC to a temporary container and replacing the water in the HC with 350 mL spring water. Spring water used throughout the study was from the same source and all fish were exposed to the same temperature (23–26ºC) and pH (7.4–7.7) water during housing. The pH of water in TC was maintained in this same range following the addition of LEO or linalool. This study was approved by the Wesleyan College Animal Care and Use Committee (ACUC protocol # HT323).

Chemicals

LEO Study. 100 µL of Lavender Essential Oil (LEO) from NOW Essential Oils (Bloomingdale, IL) composed of 100% pure *Lavender angustifolia*, was dissolved in 100% Ethanol in a 1:10 ratio, following the procedure of Can et al. (2018), a few minutes prior to beginning LEO trials. To achieve this dilution, 100 µL of LEO was pipetted into a 15 mL screw cap tube and 900 µl of Ethanol was added to the vial (achieving a 1:10 dilution of LEO in Ethanol), then shaken vigorously. This LEO solution was then added to 3 mL of water from the TC, shaken vigorously and added to the TC (containing 696 mL of spring water), immediately prior to each LEO trial. This resulted in a final LEO concentration of 1:7000. The Vehicle Control consisted of 900 µl of Ethanol added to 3.1 mL of TC water, which was shaken vigorously and added to the TC (containing 696 mL of spring water) immediately prior to each Vehicle trial. Control animals were exposed to 700 mL of water in the TC alone, without the addition of chemicals.

Linalool Study. A stock solution of 500 ppm linalool was made from linalool purchased from Sigma-Aldrich. Linalool at this concentration is soluble in water; therefore, dilutions were performed from the stock solution to achieve 60 ppm, 30 ppm, and 15 ppm in spring water.

Experimental design

LEO Study. Male Crown-Tail *Betta splendens* (CT, N = 6, labeled a-f) were exposed to male Veil Tail *Betta splendens* challengers (VT, N = 3, labeled C1-C3). Each CT acted as its own Control; responses under each experimental condition were compared to the untreated condition in a pre/post design. The Untreated, Vehicle Control and LEO conditions were repeated twice; thus, each of the six CT fish were exposed to 6 challenges per condition. The experiment was completed over a 7-week period with trials conducted every other day, each day alternating challengers. The testing containers were filled with 700 mL of spring water prior to each trial. First the CT were exposed to each challenger untreated, and these trials replicated. Next, CT were exposed to each challenger in the context of the Vehicle Control and these trials replicated. Finally, CT were exposed to challengers in the context of LEO treated water and these trials were replicated to conclude this series of experiments.

For each trial, the CT was transferred to their corresponding testing container using a fish net. The testing container was placed in a large, white Styrofoam box and the CT was allowed 2 minutes to acclimate. A challenger in its own holding container was then placed next to them, to allow visual contact, and their interactions were video recorded with an iPhone for 3 minutes. The CT was then transferred back to its holding container and the challenger was permitted 10 minutes of rest before being exposed to the next fish. This process was repeated for all CT so that on each experimental day, all CT were exposed to one challenger VT.

Leo Recovery trials. At one week, one month, two- and three-months post experiment, CT fish were again exposed to challengers, to determine if their responses had returned to pre-treatment levels. For each of these trials, each CT saw each challenger once (thus, the data represent six fish having three challenges).

Linalool Study. The linalool trials were completed by similar methods to the LEO study, consisting of presenting male Crown-Tails with a challenger and filming for 3 minutes while in the treatment condition. However, to avoid challenger fatigue, new Crown-Tails were used for each trial, so fish were exposed to each challenger only once. Four male Hellboy *Bettas* were used as challengers, one for each of the CT treatment conditions: Control, 60 ppm, 30 ppm, and 15 ppm. Experiments were replicated in three separate trials, with a total of 8 animals per treatment condition, 9 animals for Control (N = 33 animals total).

Data Analysis. Videos were observed for the presence of frontal display or caudal swing agonistic behaviors to measure aggression (Table 1). The number of behaviors were counted for each 3-minute video from each fish and tabulated. For the LEO study, mean pre-treatment behaviors were compared to those elicited in the presence of Vehicle Control (Ethanol) or LEO treatment using a repeated measures Analysis of Variance (ANOVA). For the linalool study, a one-way ANOVA was used. Mean responses were considered to be different at $\alpha < 0 .05$. Post-hoc analysis was accomplished via Tukeys HSD and means considered to be different at $\alpha < 0.05$.

Results

To assess the influence of LEO treatment on agonistic *Betta* fish behaviors, fish aggressive displays were first observed in an untreated condition (Control) to determine their responsiveness to VT challengers, followed by their response under treatment with the vehicle for solubilizing LEO (Ethanol) and then, finally, under the influence of LEO treatment. Under pre-treatment (Control) conditions, videos showed most of the male Crown-Tails were very active during the entire period of exposure to challengers (so, during both the 2-minute acclimation period and the 3-minute video recording window), consistently exhibiting aggressive behaviors towards the challenger that was present during the three-minute recording window. While only caudal swing and frontal display were used to measure aggression, other agonistic behaviors were also seen (data not shown). The behaviors observed in the Control were reiterated in the Vehicle Control; during both the acclimation period and the three-minute recording window, fish were actively engaging the challenger and multiple agonistic behaviors were recorded.

During the first 2 minutes of LEO exposure (the acclimation period provided all fish in all conditions), the *Bettas* were actively swimming and attentive to the challenger. The 3-minute recordings of the challenger interactions revealed a diminishment and eventual halt to agonistic behaviors. The LEO trial videos showed the fish were markedly less active, with little to no interest in the challenger and minimal agonistic behaviors. Some LEO treated fish exhibited slower operculum movements and fewer fin movements than either pre-treatment Control or Vehicle treated conditions (data not shown), but after removal from LEO-treated water, the CT recovered normal operculum movement and normal swimming behaviors within 10 minutes of their return to the housing containers.

The data in Figure 1 presents the average aggressive displays (frontal display or caudal swing agonistic behaviors) among untreated animals (Control), those treated with the ve-

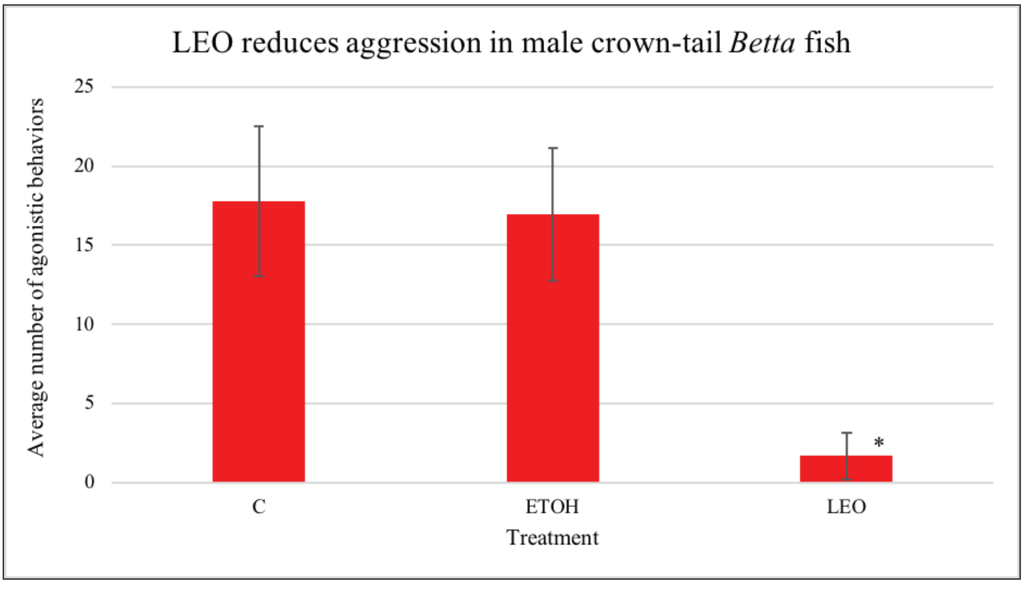

Figure 1. Treatment with LEO reduces aggression in male crown-tail *Betta* fish. The y-axis represents the mean of the agonistic behaviors exhibited during the 3-minute video recordings. The x-axis indicates the treatment condition and vertical lines represent the standard deviation of the mean for that group. The data indicate a dramatic, statistically significant, difference among mean agonistic responses (ANOVA $F(2,48) = 97.62$, $p = 1.2154E-17$) of fish in this trial. Subsequent post-hoc analysis indicates no significant difference between the Control and Vehicle Control (ETOH) mean responses (Tukey's HSD, $\alpha > 0.05$). Significant differences (*) were determined between the means of Control vs LEO and ETOH vs LEO agonistic behaviors (Tukey's HSD, $\alpha < 0.05$)

hicle for solubilizing LEO (Ethanol) and LEO-treated animals, in response to VT challengers. The data indicate a dramatic, statistically significant, difference among mean agonistic responses (ANOVA $F(2,48) = 97.62$, $p = 1.2154E\text{-}17$). Post hoc analysis indicated no difference between pre-treatment, Control responses and Ethanol treated groups (Tukey's HSD, $\alpha > 0.05$), with a significant difference between Control and LEO treatment, as well as Ethanol and LEO treatment (Tukey's HSD, $\alpha < 0.05$).

The entire experiment was repeated with the same fish (Trial 2) and Figure 2 indicates that although fish were significantly less responsive in the second trial, inhibition of agonistic behavior was reiterated with LEO treatment. The data indicate that although fish responsiveness was significantly decreased in the second trial vs the first (Trial2*, $F(1) = 9.62$, $\alpha < 0.01$), the significant inhibition of agonistic responses by LEO treatment, in comparison to either Control or Ethanol treatment, was completely reproducible in the second trial.
Challenging of the fish one week following the final LEO exposure suggested that while agonistic behaviors increased from the levels seen under LEO treatment, they did not return to pre-treatment levels. Fish were therefore assessed at one week, one month, two months and at three months following their LEO exposure, to determine if LEO effects on behavior were completely reversible.

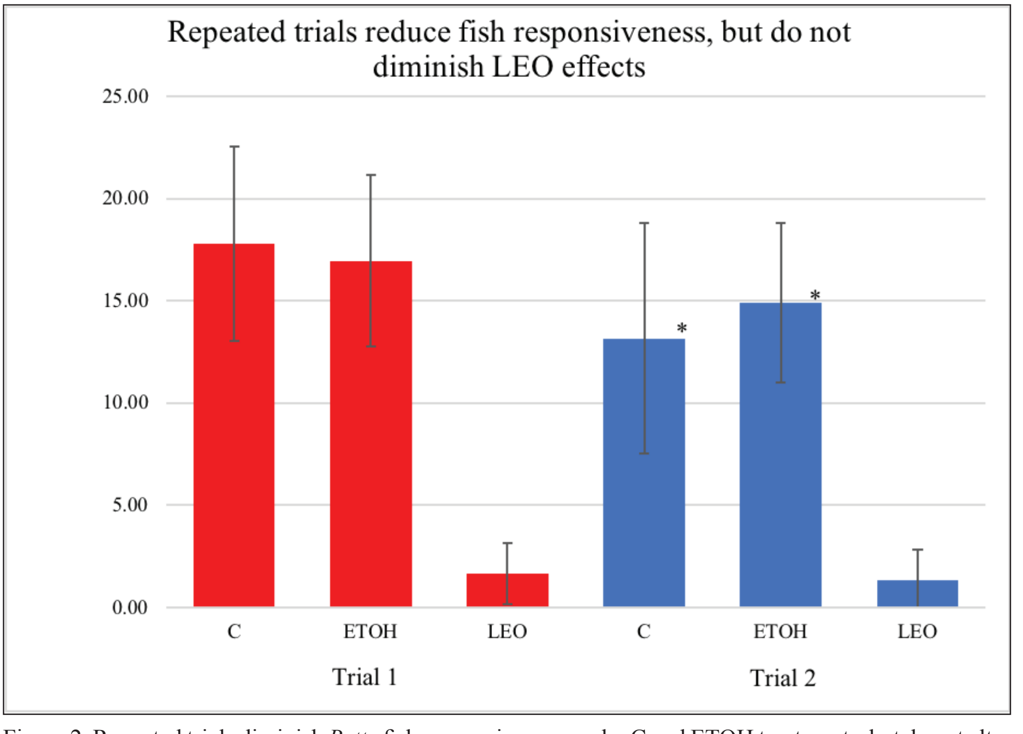

Figure 2. Repeated trials diminish *Betta* fish responsiveness under C and ETOH treatments, but do not alter LEO inhibition of agonistic behaviors. The y-axis represents the mean of the agonistic behaviors exhibited during the 3-minute trials. The x-axis indicates the treatment condition and vertical lines represent the standard deviation from the mean for that group. The * asterisks indicate a difference between the means, so C responses in trial 2 are different from C trial 1 and mean agonistic responses under ETOH treatment trial 1 is different from ETOH trial 2, (ANOVA $F(1) = 9.62$, $p = 0.0025$; Tukey's HSD $\alpha < 0.05$)); however, in each individual trial, mean responses under C and ETOH treatment were statistically indistinguishable(Tukey's HSD $\alpha > 0.05$). LEO effects were not significantly different between the two trials (Tukey's HSD $\alpha > 0.05$), but were significantly different from both C and ETOH in each trial (** Tukey's HSD $\alpha < 0.05$).

Figure 3 demonstrates an increase in *Betta* fish agonistic behaviors 1-week post LEO treatment, indicating a certain degree of reversibility. However, the aggression observed at 1-week post-trial was not equivalent to the behavior under pre-treatment (Control) conditions, meaning the effects of LEO treatment on agonistic behaviors persisted even after fish were no longer exposed to the chemical. One week post LEO treatment, the *Bettas* acknowledged their opponents and exhibited some agonistic behaviors; however, the frequency of these behaviors was much less than that displayed in the pre-treatment Control condition. To determine if animals needed more time to recover, they were assessed again at 1-month, 2-month, and 3-months post LEO treatment, to determine if agonistic behaviors would continue to increase. While there was an increase in agonistic behaviors at the 1-week post-treatment period, after that time, agonistic behaviors plateaued, and never returned to Control levels. Comparison of the means for this analysis revealed significant differences among the groups (ANOVA $F(5, 102) = 18.63$, $p = 4.10866e-13$); post hoc analysis indicates recoveries were significantly different from the Control and LEO (Tukey's HSD, $\alpha < 0.05$), but not significantly different from each other (Tukey's HSD, $\alpha > 0.05$). Thus, no additional recovery in agonistic response was made over time and all LEO-treated fish demonstrated significantly fewer agonistic responses versus Control (pretreatment) levels. Observations from the videos revealed the CT interest in their opponent was markedly decreased in comparison to that seen in the pre-treatment Control condition. This suggests that, under the treatment regimen used in these experiments, LEO effects were long-lasting and did not permit a return to normal (pre-treatment) agonistic responses.

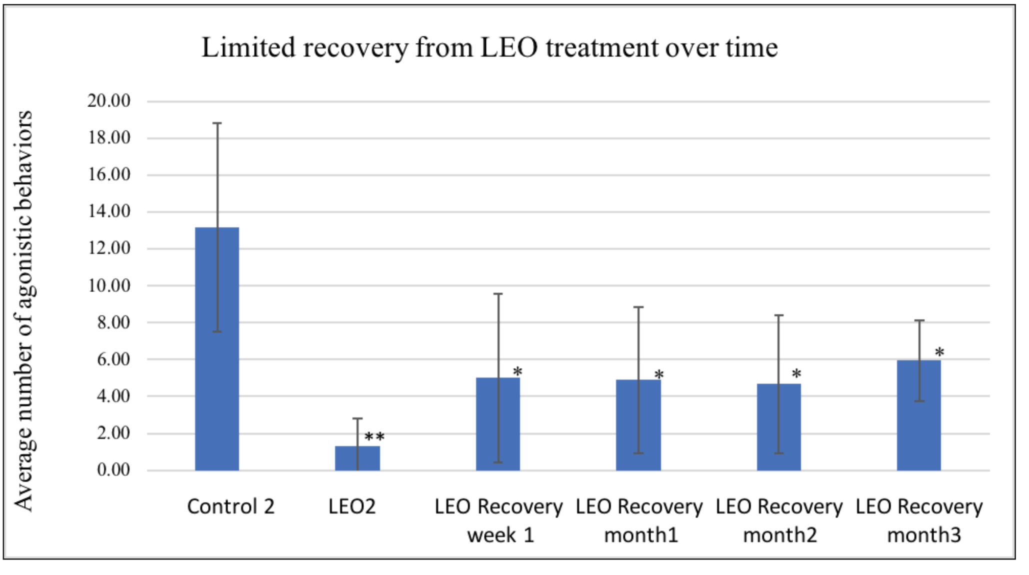

Figure 3. Partial recovery from LEO's inhibitory effects on agonistic behaviors over time. The average number of agonistic responses from Control trial 2 and LEO trial 2 groups were compared with fish Recovery at 1 week, 1 month, 2 months, and 3 months post LEO treatment. The y-axis represents the mean of the agonistic behaviors exhibited during the 3-minute trials. The x-axis indicates the treatment condition and vertical lines represent the standard deviation of the set of numbers for that group. Analysis of the data indicated a significant difference among the means (ANOVA $F(5,102) = 18.63$, $p = 4.10866E-13$); post hoc analysis indicates recovery behaviors were significantly different from the Control 2 and LEO 2 (*, Tukey's HSD, $\alpha < 0.05$) means, but not significantly different from each other (Tukey's HSD $\alpha < 0.05$).

While treatment with LEO did inhibit agonistic responses in *Betta splendens*, the formulation used was a mixture of extracted *Lavender angustifolia,* and not a purified compound. Therefore, an additional experiment with a purified preparation of one of the principal constituents of LEO, linalool, was conducted to determine if this chemical, in isolation, effected *Betta splendens* agonistic responses.

Previous studies in blue dolphin cichlids indicated an anesthetic effect of LEO at 200–400 ppm, with ready recovery (Can and Sümer 2019). Other studies, using linalool to test its anesthetic properties on juvenile carp, used treatment levels of 200–2400 ppm (Yousefi et al. 2018). As we intended to avoid anesthetizing the fish, a much lower dose of linalool was used for a preliminary dose response study, to determine if linalool treatment alone might influence *Betta* agonistic behaviors. The data in Figure 4 indicate that at both 30 ppm and 60 ppm, treatment of fish with linalool resulted in significantly fewer agonistic responses versus those exhibited by untreated, Control animals (ANOVA, $F(3,29) = 9.73$, $p = 0.0001$; Tukeys HSD $\alpha < 0.05$). Only one fish had slower opercular movements and temporary loss of equilibrium, at the 60 ppm treatment level. After removal from linalool treatment, the CT immediately exhibited normal swimming behaviors (data not shown). Thus, at the treatment level used in this experiment, it is clear that linalool alone is capable of altering agonistic behavior in *Betta splendens*, with little or no evidence of anesthetic effects.

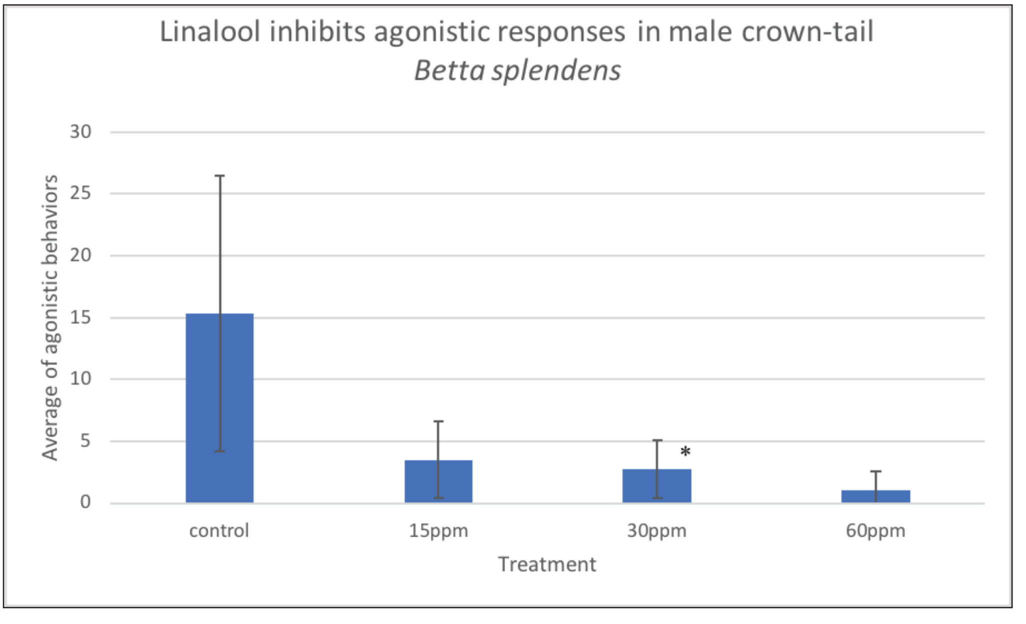

Figure 4. Linalool inhibits agonistic behaviors in *Betta splendens*. Dose response study with male CT exposed to either 15, 30 or 60 ppm linalool during challenger interactions. The y-axis indicates the mean agonistic behaviors (± SD) observed over the 3 minute video recording period for Control (N = 9) or linalool (N=8 for each treatment level) treated subjects; the x-axis indicates the treatment. The data indicate that at both 30ppm and 60ppm, treatment of fish with linalool resulted in significantly fewer agonistic responses (indicated by *) versus those exhibited by untreated, Control animals (ANOVA, $F(3, 29) = 9.73$, $p = 0.0001$; Tukeys HSD $a < 0.05$).

Discussion

In this study, lavender essential oil (LEO) significantly reduced aggressive behaviors in *Betta splendens*. The inhibition of aggressive behavior was reversible to some degree, as evidenced by the increase in aggression following recovery from LEO treatment. However, several months of testing revealed the CT did not return to their normal agonistic behaviors, suggesting this treatment may have permanent, or at least long-lasting, effects on fish behavior. Previous studies testing essential oils as anesthetics indicated the treatment was reversible, as evidenced by a recovery of equilibrium and return to normal swimming (Can and Sümer 2019; Kizak et al. 2018). It is possible that, at the level of treatment used in this study, LEO was inducing an anesthetic effect, as instances of lost equilibrium and reduced movements were observed in some animals during the treatment period. However, animals did resume normal feeding and returned to normal swimming patterns after being returned to their housing containers (data not shown). As the effect of LEO treatment on agonistic behavior was maintained for an extended period (up to 3 months), after animals had resumed months of normal swimming and feeding behaviors, it is unlikely that LEO effects on agonistic behaviors were due to anesthesia.

As a natural alternative to synthetic fish sedatives, lavender oil has been used in a limited number of different species, including blue dolphin cichlid and rainbow trout (reported in Can and Sümer 2019). However, as a mixture of different compounds, lavender extracts might have variable constituents and the observed effect in this study could have been due to an impurity introduced in the context of processing the botanical. We therefore conducted an additional experiment to isolate the effect, if any, of linalool alone on *Betta splendens* agonistic behaviors. As no similar studies have been performed in *Betta splendens*, we chose a dosage range that was well below the reported levels of anesthesia induced in fish by LEO or linalool. The data, indicating significant inhibition of agonistic responses versus Control at 30 and 60 ppm linalool, suggest that linalool alone can alter *Betta splendens* behavior. Further dose response studies, using a wider range of linalool concentrations, will be conducted in the future, to better characterize this effect.

In the context of LEO pre-treatment and Vehicle Control trials, substantive variability in agonistic behaviors was observed. It is possible that, with repeated exposure to the same challengers, the fish might adapt as they realize the challenger isn't a threat (Dzieweczynski 2012). We hypothesized that using greater numbers of CT, seeing challengers only once, might reduce this variability. The linalool experiment consisted of four challengers and new CT for each trial, to help eliminate the opponent familiarity variable. Nonetheless, as seen in Figure 4, substantive variability in agonistic behaviors was observed. Further studies, using additional measures of *Betta* behavior, might assist in better understanding this variability. *Betta splendens* are often housed in bulk at pet stores and during transportation. Both scenarios activate their stress response mechanisms, causing physiological changes which lead to corticosteroid and catecholamine release (Souza et al. 2019). Extended stress responses lead to poor health outcomes in these fish and a better understanding of factors that can ameliorate these responses will assist in maintaining healthier stocks. Lavender essential oil has lipo-solubility, permitting dispersion across biological membranes such as the blood brain barrier and central nervous system (Souza et al. 2019). Previous studies have identified linalool as an active ingredient in LEO that acts on the somatic sensory nervous system, blocking action potentials by inhibiting voltage dependent Na^+ channels, affecting neuronal excitability (Leal-Cardoso et al. 2010). These effects ultimately block sensory and motor output, resulting in depression of the central nervous system and overall behavior reduction.

Although the current study does not address a mechanism for the observed effects of LEO or linalool, additional studies might help elucidate the mechanism by which these chemicals inhibit aggressive agonistic behaviors, providing insight that might help improve the health of these organisms.

Conclusion

Using substances on animals to help control or improve behavior is a well-known practice. Essential oils are environmentally friendly, cost-effective, and very accessible, with considerable promise as sedatives, anesthetics, and, as indicated in the current study, behavior-altering substances. With more testing, the use of essential oils, such as lavender, for reducing stress and aggression in *Betta* fish, as well as other tropical fish, might provide better alternatives to the currently used artificial chemical substances.

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