Taurine modulates acute ethanol-induced social behavioral deficits and fear responses in adult zebrafish

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Highlights

- Taurine affects shoaling behavior in adult zebrafish.
- Taurine and ethanol display a temporal effect on zebrafish shoal cohesion.
- Ethanol associated with high taurine concentrations decreases social preference.
- Taurine potentiates ethanol-induced reduction in risk assessments.

Abstract

Ethanol (EtOH) is a central nervous system (CNS) depressant drug that modifies various behavioral domains (e.g., sociability, aggressiveness, and memory) by promoting disinhibition of punished operant behavior and neurochemical changes. Taurine (TAU) is a β-amino sulfonic acid with pleiotropic roles in the brain. Although exogenous TAU is found in energy drinks and often mixed with alcohol in beverages, the putative risks of mixing TAU and EtOH are poorly explored. Here, we investigated whether TAU modulates social and fear responses by assessing shoaling behavior, preference for conspecifics and antipredatory behavior of adult zebrafish acutely exposed to EtOH. Zebrafish shoals (4 fish per shoal) were exposed to water (control), TAU (42, 150, and 400 mg/L), 0.25% (v/v) EtOH alone or in association for 1 h, and their behaviors were analyzed at different time intervals (0–5 min, 30–35 min, and 55–60 min). The effects of TAU and EtOH were further tested in a social preference test and during exposure to a predator. Both EtOH and TAU co-treated fish showed a higher shoal dispersion, while TAU 400/EtOH group shoal area had a similar profile when compared to control. However, in the social preference test, TAU 400/EtOH impaired the seeking for conspecifics. Regarding fear-like behaviors, TAU-cotreated fish showed a prominent reduction in risk assessments when compared to EtOH alone. Collectively, we demonstrate that TAU modulates EtOH-induced changes in different behavioral domains, suggesting a complex relationship between social and fear-like responses.

Keywords: alcohol; shoaling behavior; antipredatory responses; taurine; zebrafish.

1. Introduction

Alcohol misuse represents a critical public health concern due to the high prevalence of alcohol-related morbidity and mortality in adults (WHO, 2014). Ethanol (EtOH) directly affects the central nervous system (CNS) and causes behavioral changes by disinhibiting the punished operant behavior and promoting cognitive deficits, which may impair threat-perception (Mitchell and Potenza, 2014). Moreover, alcohol consumption increases the risk of social and health problems leading to alterations in social behavior (e.g., sociability deficits and depressive-like behavior) (Muller et al., 2017; Naimi et al., 2003; Rosenquist et al., 2010). Since EtOH modulates brain functions involved in sociability, impulsivity, and risk assessment (Parker et al., 2014), studies related to alcohol consumption and behavior are imperative. EtOH acts in the CNS by altering various neurotransmitter systems, disrupting mitochondrial function, changing gene expression, and altering transduction-signaling pathways (Davies et al., 2003; Harper and Matsumoto, 2005; Harper and Littleton, 1990; Tong et al., 2011). Because alcohol has pleiotropic actions in the brain, interrelated neural mechanisms are likely to be involved in the pharmacological mechanisms associated with changes in sociability and critical judgment (Heinz et al., 2011).

In young adults, alcohol beverages are often consumed mixed with energy drinks, with users reporting decreased drowsiness and improved pleasure sensation (Ferreira et al., 2004a; Ferreira et al., 2004b). Taurine (TAU) is one of the main molecules present in energy drinks (Heckman et al., 2010) and its neuromodulatory function plays a key role in behavior modification (Mezzomo et al., 2018). In vertebrates, TAU can be produced endogenously and its beneficial roles in the CNS physiology include inhibitory modulation (analogous to GABA and glycine), antioxidant potential, membrane stability, osmoregulation, as well as the regulation of intracellular Ca^{2+} metabolism (Huxtable,

1992; Wu et al., 2000; Wu et al., 1992). TAU modulates complex behaviors, such as aggressiveness (Fontana et al., 2016; Oja and Saransaari, 2007), fear, and anxiety (Kong et al., 2006; Mezzomo et al., 2016; Rosemberg et al., 2012), and modifies hippocampal functions permanently (Franconi et al., 2004). Acamprosate (*N*-acetyl-homotaurine; a TAU analog) is used for treating alcoholism, and is believed to work by modulating inhibitory (GABA) and excitatory (glutamate) neurotransmitter activity that stimulate EtOH-mediated withdrawal responses (Witkiewitz et al., 2012). Importantly, TAU may also compensate EtOH effects by its neuroprotector role in the brain that includes antioxidant and membrane stabilizer activity. Moreover, because TAU and EtOH exert their role by modulating GABAergic and glutamatergic systems, the concomitant use of these molecules may counteract or even potentiate EtOH-induced behavioral changes.

Although TAU analogs exert positive effects to treat alcoholism, the influence of simultaneous TAU and EtOH consumption on both social and aversive behaviors is still poorly understood (Ferreira et al., 2006). Thus, considering that EtOH and TAU alone may affect different behavioral domains, we aimed to evaluate a potential effect of TAU and EtOH co-exposure in social and fear responses. Zebrafish are widely used in alcohol psychopharmacology, and adult specimens show behavioral sensitivity to both acute and chronic EtOH exposures that parallels with those observed in mammals following EtOH administration regimens (Gerlai et al., 2000; Muller et al., 2017). For example, adult zebrafish show concentration-dependent decreases in both social behavior (shoaling) and fear behavior (predator avoidance) following acute exposure (Gerlai et al., 2006; Parker et al., 2012). Therefore, in the present study we tested the hypothesis that TAU would affect EtOH-induced decreases in social behavior and fear-like responses in adult zebrafish. Thus, we explored how TAU, EtOH, and their concomitant exposure affect specific behavioral endpoints in different contexts and tasks, such as social behavior

(shoaling behavior and social preference tests) and antipredatory responses (predator exposure task).

2. Materials and Methods

2.1. Animals

A total of 192 wild-type zebrafish (*Danio rerio*) (4-6 months-old, ~50:50, male: female ratio, short fin strain) were obtained from a commercial supplier (Hobby Aquários, RS, Brazil). Animals were acclimatized for 15 days before the experiments under standard laboratory conditions. Water condition was set at 25 ± 2 ºC and pH 7.1, while the illumination was provided by fluorescent lamps on a 14/10 light/dark photoperiod cycle (lights on at 7:00 a.m. and off 9:00 p.m). Fish were fed twice daily with commercial flake fish food (Alcon BASIC®, Alcon, Brazil) and the water quality was monitored by commercial kits for pH, nitrite, and ammonia (Alcon BASIC®, Alcon, Brazil). Animals were maintained in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. All experiments were run in multiple days using at least three different cohorts with a randomized treatment order and fish were not originated from the same housing tank. The protocols were previously approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (process number 026/2014).

2.2. Experimental design

TAU and EtOH were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany), respectively. TAU (42, 150, and 400 mg/L) and EtOH (0.25 v/v) were added directly to the tank water concurrently or alone, and the control group was exposed to non-chlorinated water **(Fig. 1A)**. In order to asses shoaling behavior during exposure period (1 hour), 4 fish were simultaneously exposed to TAU or/and EtOH in a

final volume of 2 L (tank dimensions: 25×15×10 cm length x height x width). The same fish were later tested in the social preference test or in the predator exposure task in the absence of drugs **(Fig. 1B)**. TAU concentrations were chosen based on previous reports, where 42, 150, and 400 mg/L exerted significant neuromodulatory effects on zebrafish behavior (Fontana et al., 2016; Mezzomo et al., 2016; Rosemberg et al., 2010). EtOH concentration was chosen as described elsewhere (Fontana et al., 2016; Gerlai et al., 2000), in which 0.25% (v/v) positively modulates aggression without causing sedation in zebrafish. All behavioral tests were recorded at the same period (between 09:00 am and 4:00 pm) and animals were not fed before testing.

2.3. Shoaling behavior test

Four fish were simultaneously placed in the test tank (25×15×10 cm length x height x width) and group behavior was analyzed during the 1 h exposure period. Although zebrafish form larger shoals in their natural environment, previous data show reproducible social behavior data using four fish per shoal (Canzian et al., 2017; Green et al., 2012; Muller et al., 2017; Schmidel et al., 2014). To investigate the temporal effects of TAU and EtOH on fish behavior across the exposure period, we assessed group activity at different time intervals (T1: 0–5 min; T2: 30–35 min; T3: 55–60 min) (n = 6 shoals *per* treatment group). After recording, the apparatus was cleaned, and a new group was tested. Videos were exported to Image J 1.49 software and shoaling behavior was assessed using screenshots taken every 15 s during the 5-min trials (20 screenshots *per* trial) (Green et al., 2012; Schmidel et al., 2014). The number of social interactions was measured as described previously, which included fish proximity to conspecifics with visual contact to other member of shoal at a maximum distance of 3 body lengths (6 cm) (Canzian et al., 2017). The indexes vary from “0” (low cohesion) to “6” (complete cohesion) and the

129 area under de curve (AUC) was used to estimate the number of social interactions.
130 Screenshots were also calibrated proportional to the size of the tank to allow the
131 quantification of total inter-fish distance and shoal area using Image J 1.49 software.
132 Zebrafish vertical position was measured manually by counting the number of animals in
133 the upper half of apparatus every 15 s and data were analyzed by measuring the AUC
134 obtained per shoal. Two trained observers (inter-rater reliability > 0.90) blind to the
135 experimental condition analyzed the results.
136
137 *2.4. Social preference test*
138 Zebrafish is a social species that exhibits natural preference for conspecifics under
139 neutral and slightly aversive conditions (Saverino and Gerlai, 2008). To assess the social
140 preference, fish were placed individually in the social preference apparatus (25×15×10
141 cm length x height x width) (n = 10-12 *per* treatment group). The "n" ranged between 10-
142 12 since some animals (independently from group tested) did not respond to the
143 behavioral tasks, spending more than 80% of time immobile in the apparatus (exclusion
144 criterion). This experimental tank was virtually divided in 4 segments, where A4
145 represents the closest area to conspecifics, A3 and A2 represent transition areas, and A1
146 represents the farthest area in relation to conspecifics. In one side of the tank (A1),
147 zebrafish had visual contact with an empty aquarium, while in the other area (A4) four
148 conspecifics were placed in an identical tank as stimulus. After the exposure period,
149 zebrafish were acclimated for 30 s in the test tank. Behavioral recordings were further
150 performed for 60 s based on the protocol described previously (Gerlai et al., 2000). The
151 number of transitions and time spent in each segment were quantified using the ANY-
152 maze^{TM} software (Stoelting, CO, USA) at 30 frames/s.
153

154 *2.5. Predator exposure test*

155 Antipredatory behavior is an adaptive response exhibited spontaneously in nature
156 with a key importance for survival that may reflect aversion to a dangerous situation
157 (Csanyi and Gervai, 1986; Gerlai, 1993). Fish were individually placed in a tank
158 (25×15×10 cm length x height x width) (*n* = 10-12 per treatment group) and the
159 experiments were performed based on previous protocols (Gerlai et al., 2000; Ladu et al.,
160 2015). The "*n*" ranged between 10-12 since some animals (independently from group
161 tested) did not respond to the behavioral tasks, spending more than 80% of time immobile
162 in the apparatus (exclusion criterion). Briefly, the experimental tank was virtually divided
163 in 4 areas, in which A1 was farthest area from Oscar fish (*Astronotus ocellatus*), used as
164 a predator model, and A4 was the closest one. The other areas (A2 and A3) represent
165 transition areas. Behavioral activities were recorded for 6 min and both transitions and
166 time spent in the predator area were quantified (ANY-maze™, Stoelting, CO, USA).
167 Freezing was defined as complete immobility of fish for > 2 s with increased opercular
168 beating rate, while 'risk assessments' were defined as partial or fast (< 1 s) entries in the
169 predator area with subsequent fast movements towards the safer area of the tank (Kalueff
170 et al., 2013). Freezing duration and risk assessment episodes were manually counted by
171 two trained observers (inter-rater reliability >0.85) blind to the experimental condition.
172
173 *2.6. Statistical analyses*
174 Data normality and homogeneity of variances were performed by the
175 Kolmogorov-Smirnov and Bartlett's test, respectively. Since all data were normally
176 distributed and homoscedastic, data were expressed as means ± standard error of the mean
177 (S.E.M) and further analyzed by two-way analysis of variance (ANOVA) or by mixed 3-
178 way ANOVA followed by Student Newman Keuls multiple comparisons test when

179 necessary. The mixed 3-way ANOVA was performed using TAU and EtOH as between-
180 subjects factors plus time as the within-subjects factor when shoaling behavior was
181 assessed at different time intervals. The significance was set at $p \leq 0.05$.
182
183 **3. Results**
184 *3.1. Shoaling behavior task*
185 **Fig. 2** shows the effects of EtOH and TAU on shoaling behavior at different time
186 intervals during the exposure period. A mixed 3-way ANOVA showed a significant TAU
187 x EtOH x time interaction for shoal area ($F_{(6,80)} = 2.650$, $p = 0.021$) and animals in the
188 upper segment ($F_{(6,80)} = 2.22$, $p = 0.047$). Moreover, significant EtOH x time effects were
189 observed for inter-fish distance ($F_{(2,80)} = 6.46$, $p = 0.003$) and number of interactions
190 ($F_{(2,80)} = 8.88$, $p < 0.0005$). During the exposure period, EtOH increased the inter-fish
191 distance and reduced the number of interactions (with lower values in T2 and T3
192 compared to their respective controls). When TAU and EtOH association were analyzed,
193 TAU 42/EtOH and TAU 150/EtOH groups had increased shoal disruption in T3 when
194 compared to T1, while TAU 400/EtOH cotreatment prevented these effects. Moreover,
195 TAU 42/EtOH and TAU 400/EtOH groups increased the number of fish in the upper
196 segment **(Fig. 2)**. Representative heat maps depicting the values obtained per shoal for
197 each behavioral endpoint are shown **(Supplementary Fig. 1)**.
198
199 *3.2. Social preference test*
200 **Fig. 3** shows the effects of EtOH and TAU on the social preference. We observed
201 significant effects of TAU ($F_{(3,80)} = 2.818$, $p = 0.0443$) and EtOH ($F_{(1,80)} = 4.986$, $p =$
202 0.0284), in which TAU 400/EtOH spent less time in the conspecifics section (A4) when
203 compared to control. The number of entries to S4 did not differ among groups.

204 *3.3. Aversive responses in the predator exposure task*

205 **Fig. 4** depicts the behavioral responses observed in the predator exposure task.
206 There was a significant TAU × EtOH interaction for time spent near predator ($F_{(3,83)}$ =
207 4.865, p = 0.0036) and transitions to the predator area ($F_{(3,83)}$ = 3.024, p = 0.0341). EtOH
208 and TAU 150 alone decreased the relative time spent in predator area when compared to
209 control. Moreover, TAU 150 group showed reduced transitions to the predator area, while
210 no differences were observed in cotreated animals. Regarding the risk assessments, we
211 verified significant effects of TAU ($F_{(3,83)}$ = 24.96, p < 0.0001) and EtOH ($F_{(1,83)}$ = 5.629,
212 p = 0.0200). While EtOH deceased risk assessments, the respective behavioral endpoint
213 was significantly lower in both TAU and TAU/EtOH cotreated groups. No significant
214 effects were observed in freezing duration.

215

216 **4. Discussion**

217 The aim of this study was to examine whether TAU influences EtOH-induced
218 social behavior and fear responses in adult zebrafish. This was the first study to assess
219 how TAU affects EtOH-induced shoaling behavior at different time intervals during the
220 exposure period, thus giving the potential to uncover temporal data regarding their effects
221 on social behavior. In the shoaling test, high TAU concentrations prevented EtOH-
222 induced temporal shoal cohesion deficits, but impaired the seeking for conspecifics in the
223 social preference task. Both TAU and EtOH-exposed fish showed reduced exploration in
224 the predator area and decreased risk assessment episodes, suggesting significant effects
225 on antipredatory responses. Although TAU/EtOH cotreated animals did not change the
226 exploration in the predator area, they showed a marked reduction in the number of risk
227 assessment episodes similar to TAU-treated fish, reflecting a prominent effect of TAU
228 when compared to EtOH alone.

To our knowledge, temporal analyses of shoaling behavior revealed for the first time significant effects of TAU and EtOH alone or combined that started after 30 min of exposure. These data replicate previously described alcohol-related effects on zebrafish shoals over 60-minutes exposure, where fish presented disrupted polarization and reduced shoal cohesion (Miller et al., 2013). Although the observed effects of alcohol consumption on social domain are complex and often contradictory (Monahan and Lannutti, 2000), our data show that EtOH reduces shoal cohesion as reported elsewhere (Gerlai et al., 2000; Miller et al., 2013). Zebrafish is a suitable vertebrate model to explore the social behavior domain based on their ability to form larger shoals (Stewart et al., 2014). Thus, shoaling behavior serves as a valuable tool to assess normal and pathological social situations (Buske and Gerlai, 2011; Gerlai, 2014), in which four zebrafish in a same test tank rapidly interact with conspecifics (Schmidel et al., 2014). Since the mechanisms involved in zebrafish shoaling are strongly related to the cognitive performance and decision-making strategies (Sporns, 2010), which are modulated by TAU positively (Jia et al., 2016), this molecule may influence group behavior. Importantly, anxiolytic drugs may decrease shoaling (Hamilton et al., 2017) and behavioral links between stress, resilience, anxiety, and social behavior have been suggested in rodents subjected to different social contexts (Beery and Kaufer, 2015). People with different levels of anxiety show distinct behavior patterns and autonomic neural responses during social decision-making (Wu et al., 2013). Here, TAU 150 and TAU 400 groups showed similar temporal effects on shoal dispersion when compared to alcohol alone. Conversely, TAU 400/EtOH cotreatment abolished this response, indicating that TAU could modulate EtOH-induced shoal behavioral impairments. Low EtOH concentrations elicit anxiolytic-like responses in both rodents (Varlinskaya and Spear, 2002) and zebrafish (Baggio et al., 2017; Echevarria et al., 2011; Mathur and Guo, 2011), which may be directly related to reduced

254 social behavior. Moreover, the behavioral effects of alcohol are associated with changes
255 in various neurotransmitter systems (Banerjee et al., 2014), depending on both genetic
256 and environmental factors (Vengeliene et al., 2008). Although the precise mechanisms
257 involved in shoaling behavior are still under debate (Oliveira, 2013), the modulatory
258 effects of TAU and EtOH on different neurotransmitter pathways (e.g., GABAergic, and
259 glutamatergic systems) (Kumar et al., 2010; Roberto et al., 2004; Seo et al., 2008) may
260 play a role in the social behavior phenotypes measured here.

261 Alcohol disinhibits previously punished operant responses through the anxiolytic-
262 like effects of activation of the benzodiazepine/GABA receptors (Koob et al., 1988), and
263 this may decrease awareness of hazardous situations (Spear, 2018). Although risk
264 assessments decreased in alcohol-exposed zebrafish, they also spent less time in the
265 predator area reflecting antipredatory behaviors. Our data confirmed that 0.25% EtOH
266 (v/v) increases defensive responses in dangerous situations, as observed previously where
267 zebrafish increase jumping behavior when exposed to a robotic predator (Gerlai et al.,
268 2000). Importantly, the decreased awareness of risk commonly associated with alcohol
269 effects in humans, is observed following 1% EtOH (v/v) exposure in zebrafish, suggesting
270 a concentration-dependent response (Gerlai et al., 2000). In vertebrates, the mechanisms
271 suggested for alcohol-induced effects involve the antagonism of glutamatergic NMDA
272 receptors, as well as increased dopamine and serotonin release at lower concentrations,
273 while higher doses positively activates $GABA_A$ and $GABA_B$ receptors inducing sedation
274 (Banerjee, 2014; Krystal et al., 2003). Thus, EtOH modulates awareness in dangerous
275 situations and social behaviors possibly due to its action on different behavioral domains
276 (Oliveira et al., 2013; Parker et al., 2014; Tran et al., 2016a, b).

277 Regarding the fear-like responses, TAU showed a prominent decrease in risk
278 assessments when compared to EtOH alone. Additionally, fish exposed to 150 mg/L TAU

showed reduced transitions and time spent in the predator area. Antipredatory responses serve as valuable tools for translational studies aiming to explore abnormally overstated or misdirected fears in humans. Furthermore, the neurobiological mechanisms underlying antipredatory patterns are predictably related to those involved in abnormal human fear responses (Gerlai, 2010). Thus, we suggest that TAU may increase antipredatory behaviors in aversive contexts. Since risk assessment responses in TAU/EtOH groups were similar than those observed in TAU-treated fish, we suggest that TAU influences antipredatory behaviors in aversive contexts, exerting a complex role in different behavioral domains.

Albeit no significant interaction was observed in the social preference test, TAU 400/EtOH decreased the seeking for conspecifics. Moreover, although TAU did not modulate the effects of EtOH in some behavioral endpoints, this molecule seems to rescue the effects of EtOH when the number of interactions was assessed in the shoaling behavior test. Different tasks may elicit distinct behavioral phenotypes, which can result in various responses at a same behavioral domain depending on the context. For example, in the shoaling behavior test, fish can interact with conspecifics freely allowing a proper quantification of group formation, while the social preference task measures the approach for their conspecifics in fish placed individually in the test tank (Pham et al., 2012). TAU displays a concentration-dependent effect on anxiety-like behavior, locomotion, exploration, and aggression following acute EtOH exposure (Fontana et al., 2016; Rosemberg et al., 2012). Moreover, TAU counteracts EtOH-induced neurotoxicity by decreasing brain alcohol levels and preventing locomotor impairments (Rosemberg et al., 2012). For instance, the mechanisms underlying the behavioral responses observed in TAU/EtOH groups are generally attributed to the neuromodulatory role of TAU in the brain as a $GABA_A$ agonist (Ananchaipatana-Auitragoon et al., 2015; Rosemberg et al.,

2010; Taranukhin et al., 2009; Taranukhin et al., 2010). Although TAU acts as $GABA_A$ and glycine receptors agonist, this molecule also has important pleiotropic actions (e.g., antioxidant activity, osmoregulation, and membrane stabilizer) (Huxtable, 1992; Mezzomo et al., 2018). These functions help protecting against stress-related damage in the brain and are associated to the neuroprotective role of TAU in the CNS. Additionally, the activation of both $GABA_A$ and $GABA_B$ receptors by EtOH is not the only mechanism related to alcohol-induced behavioral alterations (Davies, 2003). EtOH also increases serotonin and dopamine levels in the brain, contributing to the biphasic effects of alcohol in zebrafish (Chatterjee and Gerlai, 2009). Although TAU and EtOH may cause similar behavior alterations depending on concentration, various mechanisms associated to the neurobehavioral effects of TAU have been postulated. Nonetheless, future studies are required to elucidate the mechanisms underlying the behavioral responses observed following TAU/EtOH association.

5. Conclusion

In summary, concurrently administered TAU and EtOH may influence zebrafish social behavior and fear-like responses by abolishing temporal EtOH-induced shoal disruption and attenuating antipredatory responses following acute alcohol exposure. Our data suggest a complex effect on different behavioral domains and thus, caution should be taken with their simultaneous consumption. Importantly, more studies are necessary to investigate how TAU and EtOH association acts in the CNS and modulates zebrafish neurobehavioral phenotypes.

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Figure Captions

Fig. 1. Experimental design and behavioral tasks. **(A)** Schematic representation of the experimental groups. **(B)** Behavioral tests used to assess shoaling behavior during exposure period, social preference, and antipredatory responses. Four animals were exposed to water (control), 0.25 (v/v) EtOH, and TAU (42, 150, and 400 mg/L) alone or cotreated with TAU and EtOH for 1 h. Shoaling behavior was assessed at different time intervals and, afterwards, two animals were randomly tested in the social preference test while the other fish were submitted to the predator test. A1 is the farthest segment from conspecifics/predator, A2 and A3 are transition areas, while A4 is the closest area from conspecifics/predator.

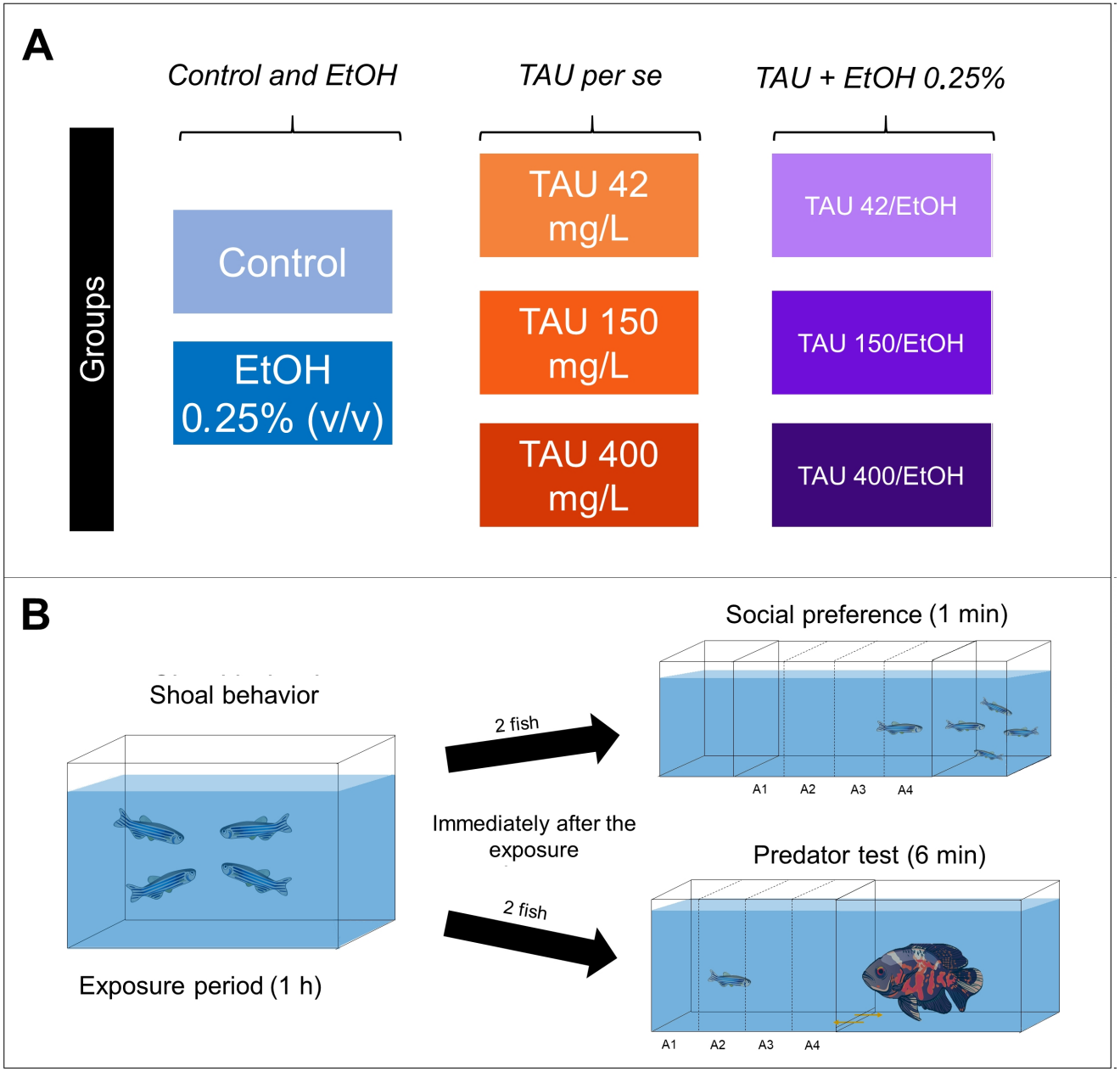
Fig. 2. Effects of TAU, EtOH and their association on shoaling behavior during 1 h of exposure. Group activity was assessed at different time blocks (T1: 0–5 min; T2: 30–35 min; T3: 55–60 min). Data were represented as mean ± S.E.M. and analyzed by mixed 3-way ANOVA followed by Student Newman Keuls multiple comparisons test when necessary. The mixed 3-way ANOVA was performed using TAU and EtOH as between-subjects factors plus time as the within-subjects factor. Different letters indicate statistical differences across time within groups, whereas the asterisks reveal statistical significances of a certain time period compared to its respective control ($p < 0.05$, $n = 6$ per group).

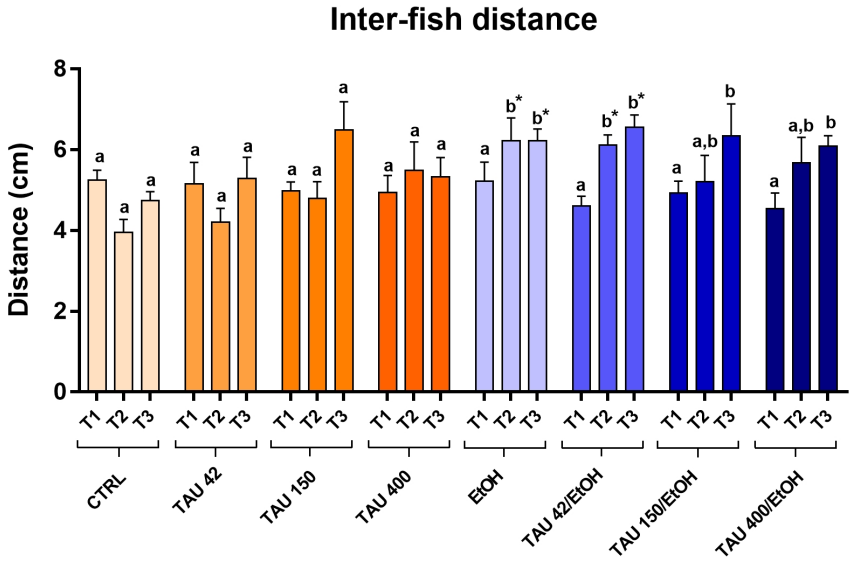
Fig. 3. Effects of TAU, EtOH and their association on social preference task. Data were represented as mean ± S.E.M. and analyzed by two-way ANOVA, following by Student

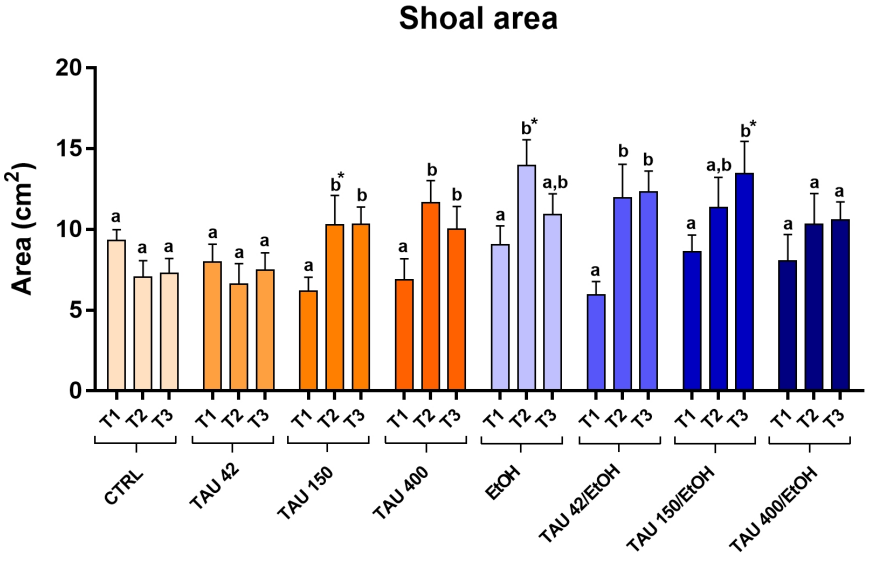
Newman Keuls multiple comparisons test when necessary. Different letters indicate statistical differences among group ($p < 0.05$, n = 10–12 per group).

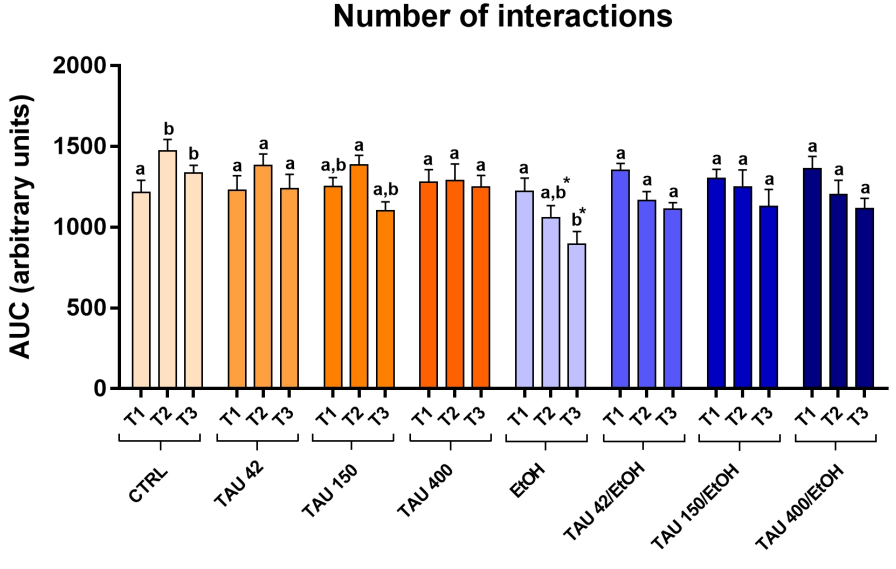
Fig. 4. Effects of TAU, EtOH and their association on antipredatory responses in zebrafish subjected to the predator exposure test. Data were represented as mean ± S.E.M. and analyzed by two-way ANOVA, following by Student Newman Keuls multiple comparisons test when necessary. Different letters indicate statistical differences among group ($p < 0.05$, n = 10–12 per group).

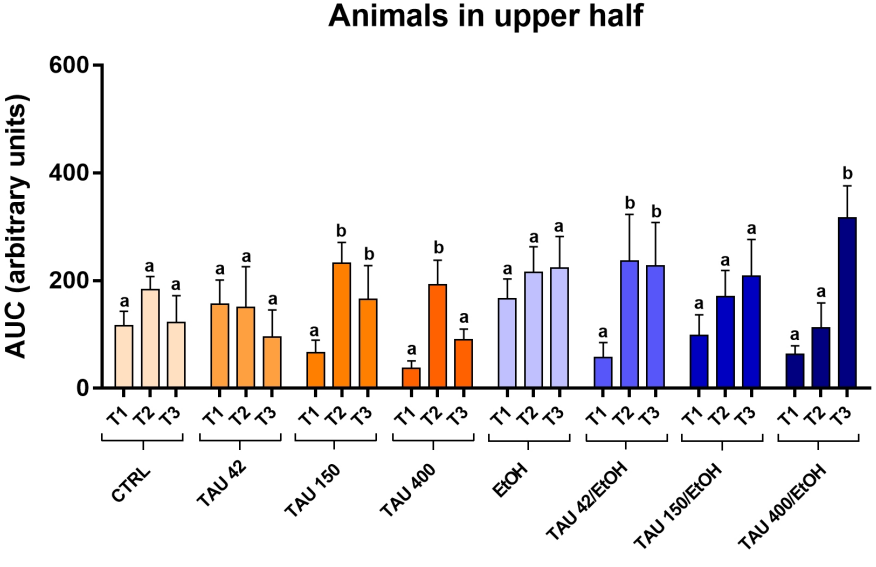
Supplementary Fig. 1. Representative heat maps showing the behavioral effects of TAU, EtOH and their association on zebrafish shoals at different time intervals during the exposure period. Values obtained for each behavioral endpoint measured (inter-fish distance, blue; shoal area, orange; number of interactions, green; animals in upper half, purple) using independent shoals (S1–S6, n = 6 per group) are depicted. A more intense color indicates higher values.

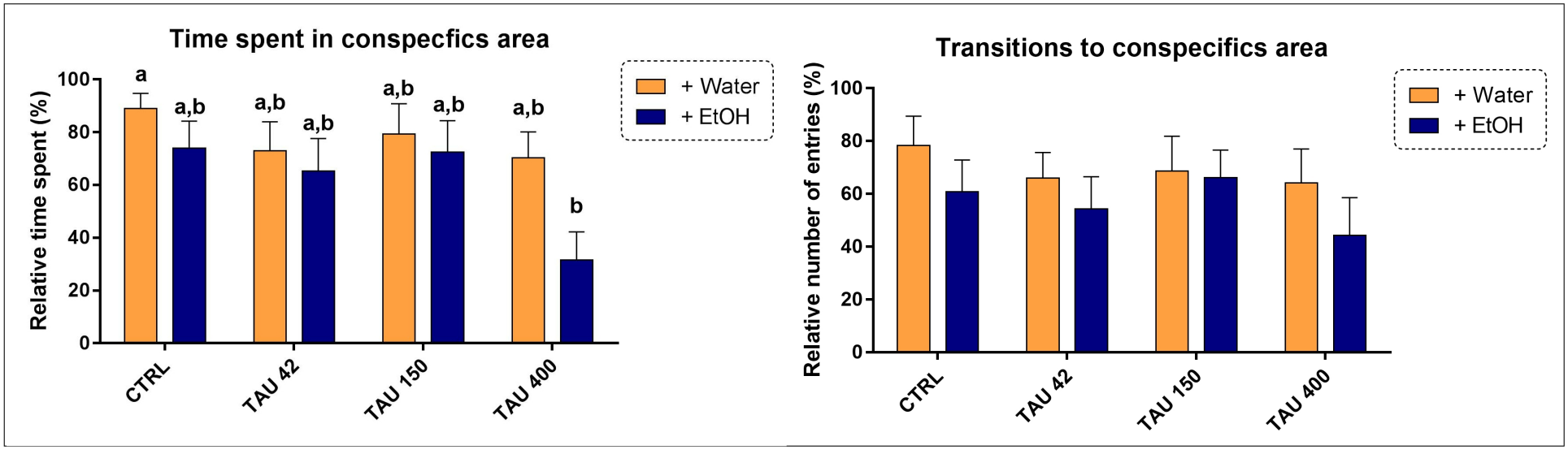

A
Control and EtOH
TAU per se
TAU + EtOH 0.25%
Groups
Control
EtOH 0.25% (v/v)
TAU 42 mg/L
TAU 150 mg/L
TAU 400 mg/L
TAU 42/EtOH
TAU 150/EtOH
TAU 400/EtOH
B
Shoal behavior
Exposure period (1 h)
2 fish
Immediately after the exposure
2 fish
Social preference (1 min)
A1
A2
A3
A4
Predator test (6 min)
A1
A2
A3
A4

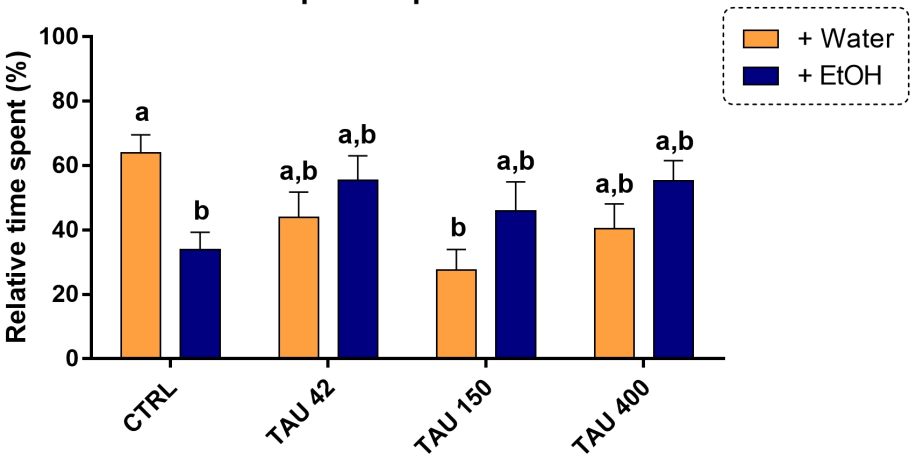

Inter-fish distance
Distance (cm)
0
2
4
6
8
T1 T2 T3
CTRL
TAU 42
TAU 150
TAU 400
EtOH
TAU 42/EtOH
TAU 150/EtOH
TAU 400/EtOH

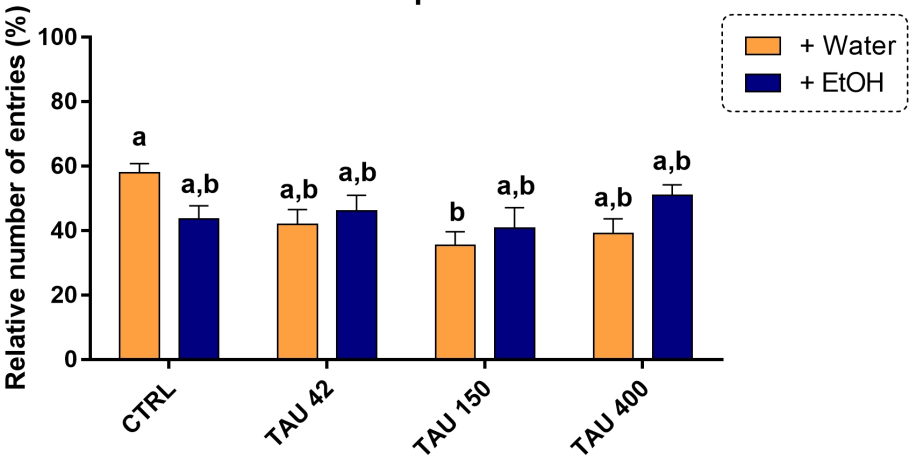

Shoal area
Area (cm2)
0
5
10
15
20
T1 T2 T3
CTRL
TAU 42
TAU 150
TAU 400
EtOH
TAU 42/EtOH
TAU 150/EtOH
TAU 400/EtOH

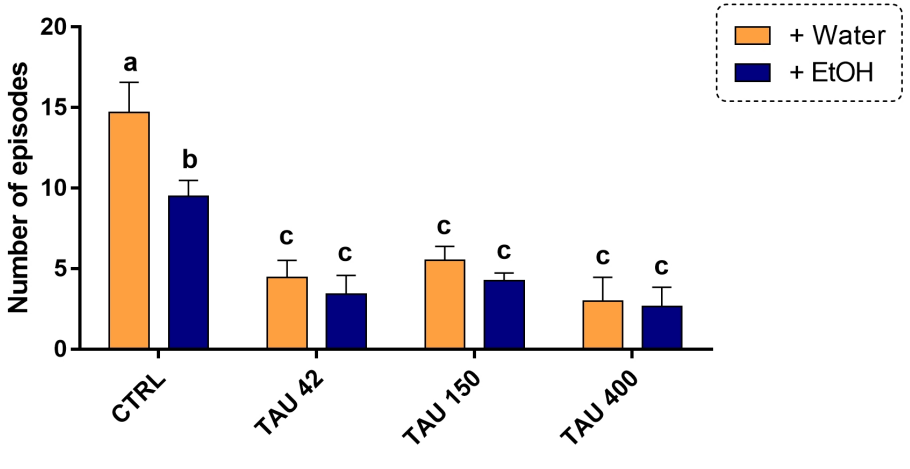

Number of interactions
AUC (arbitrary units)
0
500
1000
1500
2000
T1 T2 T3
CTRL
TAU 42
TAU 150
TAU 400
EtOH
TAU 42/EtOH
TAU 150/EtOH
TAU 400/EtOH

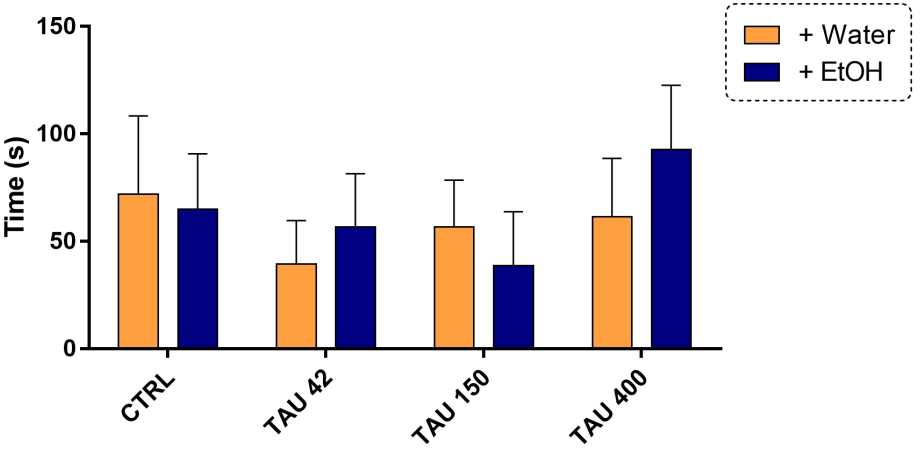

Animals in upper half
AUC (arbitrary units)
0
200
400
600
T1 T2 T3
CTRL
TAU 42
TAU 150
TAU 400
EtOH
TAU 42/EtOH
TAU 150/EtOH
TAU 400/EtOH

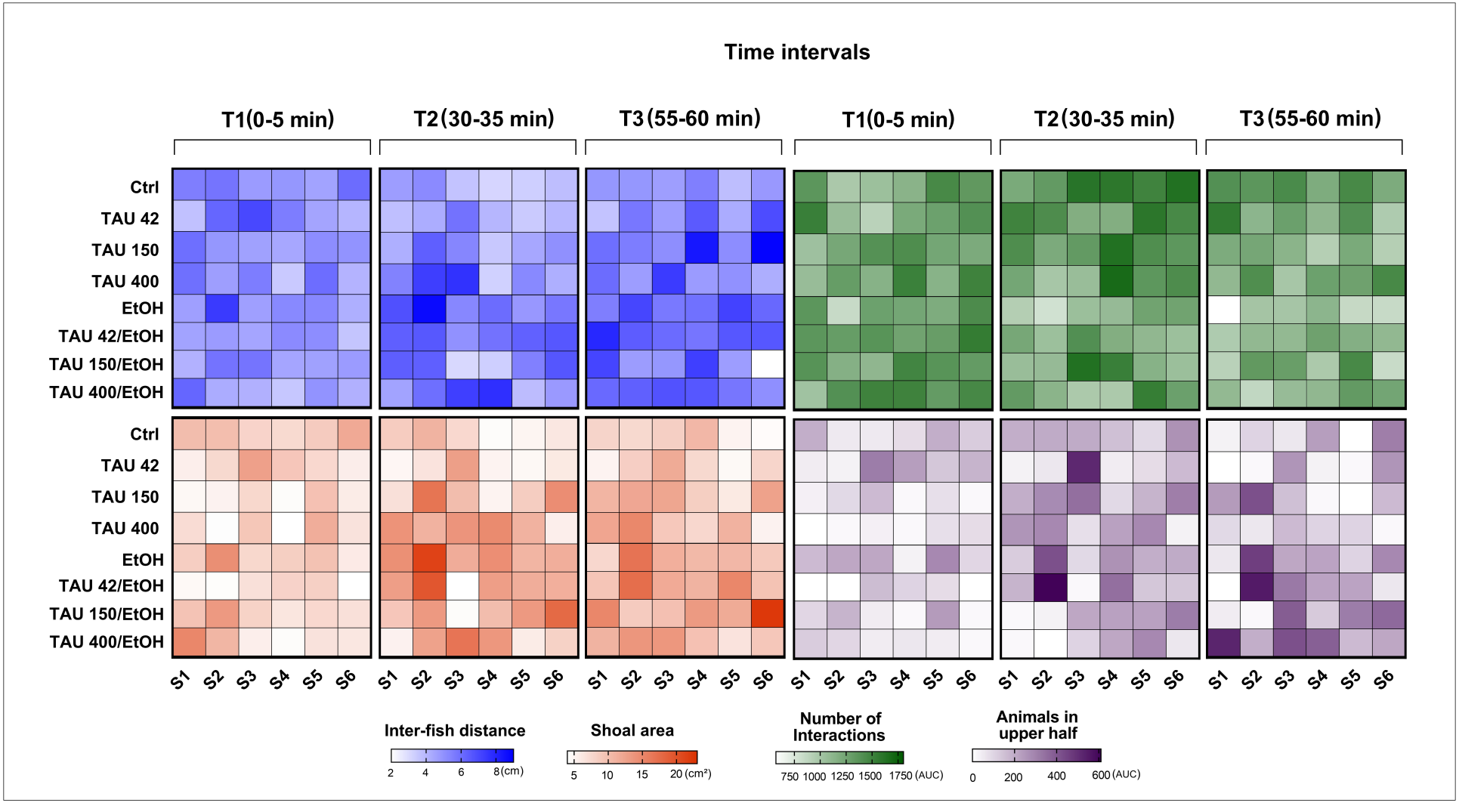

Time spent in conspecfics area
Relative time spent (%)
100
80
60
40
20
0
a
a,b
a,b
a,b
a,b
a,b
a,b
b
CTRL
TAU 42
TAU 150
TAU 400
+ Water
+ EtOH
Transitions to conspecifics area
Relative number of entries (%)
100
80
60
40
20
0
CTRL
TAU 42
TAU 150
TAU 400
+ Water
+ EtOH


Time spent in predator area
Relative time spent (%)
100
80
60
40
20
0
a
b
a,b
a,b
b
a,b
a,b
a,b
CTRL
TAU 42
TAU 150
TAU 400
+ Water
+ EtOH


Transitions to predator area
Relative number of entries (%)
100
80
60
40
20
0
a
a,b
a,b
a,b
b
a,b
a,b
a,b
CTRL
TAU 42
TAU 150
TAU 400
+ Water
+ EtOH


Risk Assessment
Number of episodes
20
15
10
5
0
a
b
c
c
c
c
c
c
CTRL
TAU 42
TAU 150
TAU 400
+ Water
+ EtOH


Freezing behavior
Time (s)
150
100
50
0
CTRL
TAU 42
TAU 150
TAU 400
+ Water
+ EtOH


Time intervals
T1(0-5 min)
T2(30-35 min)
T3(55-60 min)
T1(0-5 min)
T2(30-35 min)
T3(55-60 min)
Ctrl
TAU 42
TAU 150
TAU 400
EtOH
TAU 42/EtOH
TAU 150/EtOH
TAU 400/EtOH
S1 S2 S3 S4 S5 S6
Inter-fish distance
2 4 6 8(cm)
Shoal area
5 10 15 20(cm²)
Number of Interactions
750 1000 1250 1500 1750(AUC)
Animals in upper half
0 200 400 600(AUC)