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COMPARATIVE EVALUATION OF THE EFFECTS OF ANXIOLYTICS OF THE BENZODIAZEPINE AND NON-BENZODIAZEPINE CLASSES IN *DANIO RERIO*

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Abstract. ***Introduction.*** *Danio rerio* represents one of the most promising animal models for studying normal and pathological brain functions. Previously, the central effects of benzodiazepine anxiolytics have been demonstrated in *Danio rerio*. However, studies investigating the effects of non-benzodiazepine anxiolytics have not been sufficiently developed. ***Objective.*** Comparative evaluation of the effects of benzodiazepine and non-benzodiazepine anxiolytics in *Danio rerio*. ***Materials and methods.*** A novel tank diving test (stress-induced novelty test) was employed. Fish were initially placed in a beaker containing a dissolved pharmacological substance (or water as control), and subsequently transferred to an observation aquarium for 6 minutes. Behavioral parameters such as trajectory, distance traveled, frequency of movements into the upper part of the aquarium, time spent in the lower part, and the frequency and duration of freezing episodes were automatically recorded. Benzodiazepine anxiolytics (diazepam, tofisopam) and the non-benzodiazepine anxiolytic hydroxyzine were used at two doses: 0.5 mg/L and 1 mg/L. ***Results.*** In the novel tank test, *Danio rerio* spent most of the time in the bottom region of the aquarium, showing limited movements with frequent freezing episodes. Both frequency and duration of freezing, as well as the time spent at the bottom of the aquarium, were significantly high throughout the test period, without notable temporal changes. Administration of anxiolytics produced a typical anxiolytic effect characterized by increased time spent in the upper region, reduced frequency and duration of freezing episodes, decreased time spent in the lower part of the tank, and increased number of movements into the upper aquarium region. However, the trajectory length after administration of benzodiazepine anxiolytics remained unchanged or decreased (tofizopam), whereas it increased after the administration of the non-benzodiazepine anxiolytic hydroxyzine. Furthermore, the swimming pattern differed significantly: non-benzodiazepine anxiolytics induced uniform swimming activity across all sectors of the aquarium compared to benzodiazepine anxiolytics. ***Conclusion.*** Administration of the tested anxiolytics in *Danio rerio* induced typical anxiolytic effects. Nevertheless, significant differences in trajectory length and swimming patterns were observed between benzodiazepine and non-benzodiazepine anxiolytics. The novel tank diving test in *Danio rerio* demonstrates high selectivity and appears suitable for preclinical studies related to psychiatric and neurological disorders.

Keywords: *Danio rerio*, behavior, novel tank test, anxiolytics

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СРАВНИТЕЛЬНАЯ ОЦЕНКА ЭФФЕКТОВ АНКСИОЛИТИКОВ БЕНЗОДИАЗЕПИНОВОГО И НЕБЕНЗОДИАЗЕПИНОВОГО РЯДОВ У *DANIO RERIO*

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Резюме. ***Актуальность.*** Одной из наиболее перспективных моделей для проведения исследований функций головного мозга в норме и при патологии является *Danio rerio*. Ранее у *Danio rerio* показано центральное действие анксиолитиков бензодиазенинового ряда. В то же время исследование анксиолитиков небензодиазепинового ряда не получило должного развития. ***Цель*** — провести сравнительную оценку эффектов анксиолитиков бензодиазепинового и небензодиазепинового рядов у *Danio rerio*. ***Материалы и методы.*** Использовали тест стресса новизны: рыбу помещали сначала в мерный стакан с растворенным фармакологическим веществом (или водой), затем в просмотровый аквариум на 6 минут, где автоматически регистрировали траекторию движения, длину пути, число перемещений в верхнюю часть аквариума, время пребывания в нижней части аквариума, число и время паттерна «фризинг». Использовали бензодиазепиновые транквилизаторы диазепам, тофизопам и небензодиазепиновые транквилизаторы на примере гидроксизина в двух дозах: 0,5 мг/л и 1 мг/л. ***Результаты.*** В тесте новизны *Danio rerio* большую часть времени находились в нижней части просмотрового аквариума и совершали небольшие перемещения. При этом часто наблюдали фризинг, число и время которого за опыт были достаточно велики, как и время пребывания рыбы в нижней части аквариума. Динамика времени пребывания рыбы в нижнем и верхнем отсеках практически не изменялась. Введение транквилизаторов вызывало типичный анксиолитический эффект: подъем в верхнюю часть аквариума, снижение числа и времени паттерна «фризинг», снижение времени пребывания рыбы в нижней части аквариума, повышения числа перемещений в верхнюю часть аквариума. В то же время длина траектории после введения бензодиазепиновых транквилизаторов не изменялась или снижалась (тофизопам), а после введения небензодиазепиновых транквилизаторов (гидроксидин) повышалась. Картина траектории движения рыб также отличалась: небензодиазепиновые транквилизаторы вызывали равномерное плавание практически по всем секторам аквариума, в отличие от действия бензодиазепиновых транквилизаторов. ***Заключение.*** Таким образом, введение исследуемых транквилизаторов у *Danio rerio* вызывает типичный анксиолитический эффект. В то же время длина и картина траектории у рыб значительно отличается при сравнительной оценке эффектов анксиолитиков бензодиазепинового и небензодиазепинового рядов. Сделан вывод о высокой селективности теста новизны у *Danio rerio* для проведения доклинических исследований, связанных с психиатрическими и неврологическими расстройствами.

Ключевые слова: *Danio rerio*, поведение, стресс новизны, транквилизаторы

INTRODUCTION

Preclinical trials are based on experimental models. For many years, mammals were used for modelling brain function disorders [1]. Recent studies show a rapid increase in animal species diversity in the study of pharmacological agents. The *Danio rerio* fish is one of the most promising models for conducting studies on brain functions [2, 3]. Functions of its forebrain include complex cognitive abilities, mailnly, due to the development of subcortical neural circuits [4, 5]. *Danio rerio* has all main neurotransmitters, receptors and transporters, which are necessary for treating brain disorders in humans [6]. The neurochemical brain basis is largely similar across vertebrates and suggests modelling the search for treatment methods in lower taxonomic groups as well [3]. This allows scientists to consider *Danio rerio* as a candidate for inclusion in preclinical research protocols related to psychiatric and neurological disorders [1]. There are examples of using *Danio rerio* for modelling affective disorders [7] and depression [8]. The effect of benzodiazepine anxiolytics in *Danio rerio* has been demonstrated [9]. At the same time, the study of non-benzodiazepine anxiolytics in fish remains underdeveloped.

It was shown that the effect of benzodiazepine anxiolytics in *Danio rerio* led to an increase in stress hormones, e.g. cortisol [10]. The effects of stress on behaviour in *Danio rerio* can be investigated in a number of tests, such as the light–dark [11], T-maze [12], open field [13], and novelty stress [14]. The novelty stress test is used to study anxiety-phobic reactions in *Danio rerio* [14, 15]. During the test, fish demonstrate stable behaviour responses under the influence of novelty stress [14]. Such behaviour test is based on the instinct to seek protection from an unfamiliar environment by diving to the bottom, freezing (immobilizing), and reducing motor and exploratory activity. After adaptation (acclimation) to the new environment, motor activity increases, freezing decreases and the number of transitions to the upper half of the aquarium increases [16]. Traditionally, similar behaviour is analysed in the open-field test in studies on anxiety in rodents [17]. In the novelty stress test, *Danio rerio* is first placed in a measuring container containing the dissolved pharmacological agent (or water) and then in a small viewing aquarium, where motor activity, freezing, the number of transitions to the upper half of the aquarium and the time spent in it are recorded [18]. The behaviour of *Danio rerio* was recorded using computer-assisted video tracking. This method allows for a more detailed and highly accurate analysis of the effects of pharmacological agents [10]. We have previously described the anxiolytic effect of the tranquilizer Phenazepam in *Danio rerio* [20, 21].

AIM

The aim of the study was to conduct a comparative pharmacological analysis of the effects of benzodiazepine and non-benzodiazepine anxiolytics in *Danio rerio*.

MATERIAL AND METHODS

Animal Selection. In the study, 204 sexually mature *Danio rerio* fish (Aqua Piter company, wild-type), aged 6–8 months, were used after two weeks of adaptation to 40-liter aquariums, with 20–30 fish in each at a water temperature of 25–27 °C. The animals were kept under standard lighting conditions (from 8:00 to 20:00) at a room temperature of 22±2 °C and fed twice a day with standard Tetramin tropical flakes.

Novelty Stress Test. For the experiments, a trapezoidal viewing aquarium with a volume of 1.5 l, 15×7 cm, 22 cm long at the bottom and 28 cm long at the top of the aquarium was used. This type of aquarium is used for testing anxiety-phobic reactions in *Danio rerio* [15]. The trapezoidal shape of the aquarium serves to minimise lateral movements of fish and to freely observe vertical and horizontal movements. Since this behavioural test is based mainly on the instinct to seek protection from an unfamiliar environment by diving to the bottom [19], the aquarium was divided by a line into two equal parts — the upper and lower. The fish were first placed in a 200 ml beaker containing a dissolved pharmacological substance (or water) for 5 minutes. Then, the fish were placed in a pre-test aquarium containing water (10×10×10 cm) for 5 minutes and then in the observation aquarium for 6 minutes. Movement activity (track length), the number of times the fish moved to the upper and lower halves of the aquarium, and the time spent in halves were recorded. Automatically, during the test, the number and duration of freezing episodes (immobilization) were registered, which are usually observed during novelty stress and reflect the animal's anxiety level. The behaviour was automatically registered using the Noldus EthoVision XT7 system, allowing the researcher to view video tracks of fish. The system allows both digital data collection and visual monitoring of the video track. The entire observation period was continuously recorded, with the program recording the movement trajectory.

Pharmacological Substances. To perform pharmacological analysis, benzodiazepine tranquilizers, such as Diazepam (Sibazon) from Dalhimfarm OJSC 5 mg/ml in two doses — 0.5 and 1 mg/L — and Tofisopam (Grandaxin) from EGIS Pharmaceuticals 50 mg in two doses — 0.5 and 1 mg/L were used. Non-benzodiazepine tranquilizers were also used for the analysis, such as Hydroxyzine (Atarax) from UBC Pharma 25 mg in two doses — 0.5 and 1 mg/L.

RESULTS

Study of the action of benzodiazepine tranquilizers. All studied substances were used at two doses: 0.5 and 1 mg/L. Diazepam at a dose of 0.5 mg/L compared to the control group was significantly different in the indicator "number of movements to the upper part of the aquarium" (12.8±0.5 (p ≤0.05) and 5.8±1.2, respectively). Diazepam at a dose of 1 mg/L showed statistically significant differences with the control group in the number of freezing episodes 21.2±1.3 s (p ≤0.05) vs. 47.8±4.9 s, number of movements to the upper part of the aquarium 19.4±1.1 (p ≤0.05) vs. 5.8±1.2, respectively.

Tofisopam at a dose of 0.5 mg/L showed statistically significant differences compared to the control group in all indicators. Specifically, the number of freezing episodes decreased from 47.8±4.9 to 23±3.6 (p ≤0.05), the time of freezing episodes decreased from 23.9±2.5 sec to 11.5±1.8 s (p ≤0.05), the trajectory length decreased from 1365±57.6 cm to 1057±9.9 cm (p ≤0.05), the time spent in the lower part of the aquarium decreased from 47.7±9.3 s to 263±3.2 s (p ≤0.05), and the number of movements to the upper part of the aquarium increased from 5.8±1.2 to 35±2.7 (p ≤0.05). At a dose of 1 mg/L, Tofisopam, compared with the control group, showed statistically significant differences only in the trajectory length indicator: 776±65.9 cm (p ≤0.05) and 1365±57.6 cm, respectively. The above data are presented in Table 1.

Study of the action of non-benzodiazepine tranquilizers. In the experiments, non-benzodiazepine tranquilizer Hydroxyzine was used, which at a dose of 0.5 mg/L, compared with the control group, showed statistically significant differences in all compared criteria. The number of freezing episodes decreased from 47.8±4.9 to 24.5±5.6 (p ≤0.05), the time of freezing episodes decreased from 23.9±2.5 s to 12.2±2.8 s (p≤0.05), the trajectory length increased from 1365±57.6 cm to 1867.4±118.2 cm (p ≤0.05), the time spent in the lower part of the aquarium decreased from 347.7±9.3 s to 260.7±15.4 s (p ≤0.05), and the number of transitions from the lower part of the aquarium to the upper part increased from 5.8±1.2 to 51.6±7 (p ≤0.05).

Hydroxyzine at a dose of 1 mg/L significantly differed from the control group only in the "time in the lower part of the aquarium" parameter. Here, against the background of the introduction of Hydroxyzine, the fish reduced the stay in the lower part of the aquarium from 347.7±9.3 s in the control group to 295.4±10.8 s (p ≤ 0.05) in the Hydroxyzine 1 mg/L group, and also increased the number of transitions from the lower part of the aquarium to its upper part against the background of the introduction of Hydroxyzine 1 mg/L — 27±6.5 times (p ≤ 0.05) compared to the control group — 5.8±1.2 times. The data are presented in Table 2.

DISCUSSION

These studies have shown that the response of *Danio rerio* to being introduced into a novel observation tank exhibits a typical pattern of behaviour. In response to the unfamiliar environment, the fish initially dove to the bottom,

Table 1

Effects of benzodiazepine tranquilizers on the behavior of *Danio rerio* in the novelty test

Таблица 1

Влияние бензодиазепиновых транквилизаторов на поведение *Danio rerio* в тесте новизны

Группа / Group	Количество фризингов, n / Number of freezing episodes, n	Время фризинга, с / Freezing duration, s	Длина траектории, см / Trajectory length, cm	Время в нижней части аквариума, с / Time spent in bottom of aquarium, s	Количество перемещений в верхнюю часть аквариума, n / Number of transitions to upper part of aquarium, n
Контроль / Control	47,8±4,9	23,9±2,5	1365±57,6	347,7±9,3	5,8±1,2
Диазепам 0,5 мг / Diazepam 0.5 mg	33,2±2,2	16,6±1,1	1192,2±27,7	328±1,6	12,8±0,5*
Диазепам 1 мг / Diazepam 1 mg	21,2±1,3*	10,6±0,7*	1468,8±15,3	319,8±2,6	19,4±1,1*
Тофизопам 0,5 мг / Tofisopam 0.5 mg	23±3,6*	11,5±1,8*	1057±9,9*	263±3,2*	35±2,7*
Тофизопам 1 мг / Tofisopam 1 mg	34±2,4	17±1,2	776±65,9*	330,5±6,6	12,6±2,5*

* p ≤0,05 —relative to the control group, n=10. / p ≤0,05 — относительно группы контроля, n=10.

Table 2

Effects of the non-benzodiazepine tranquilizer hydroxyzine on the behavior of *Danio rerio* in the novelty test

Таблица 2

Влияние небензодиазепинового транквилизатора Гидроксизина на поведение *Danio rerio* в тесте новизны

Группа / Group	Количество фризингов, n / Number of freezing episodes, n	Время фризинга, с / Freezing duration, s	Длина траектории, см / Trajectory length, cm	Время в нижней части аквариума, с / Time spent in bottom of aquarium, s	Количество перемещений в верхнюю часть аквариума, n / Number of transitions to upper part of aquarium, n
Контроль / Control	47,8±4,9	23,9±2,5	1365±57,6	347,7±9,3	5,8±1,2
Гидроксизин 0,5 мг / Hydroxyzine 0.5 mg	24,5±5,6*	12,2±2,8*	1867,4±118,2*	260,7±15,4*	51,6±7*
Гидроксизин 1 мг / Hydroxyzine 1 mg	36,4±7,5	18,2±3,7	1449±88,1	295,4±10,8*	27±6,5*

* p ≤0,05 —relative to the control group, n=10. / p ≤0,05 — относительно группы контроля, n=10.

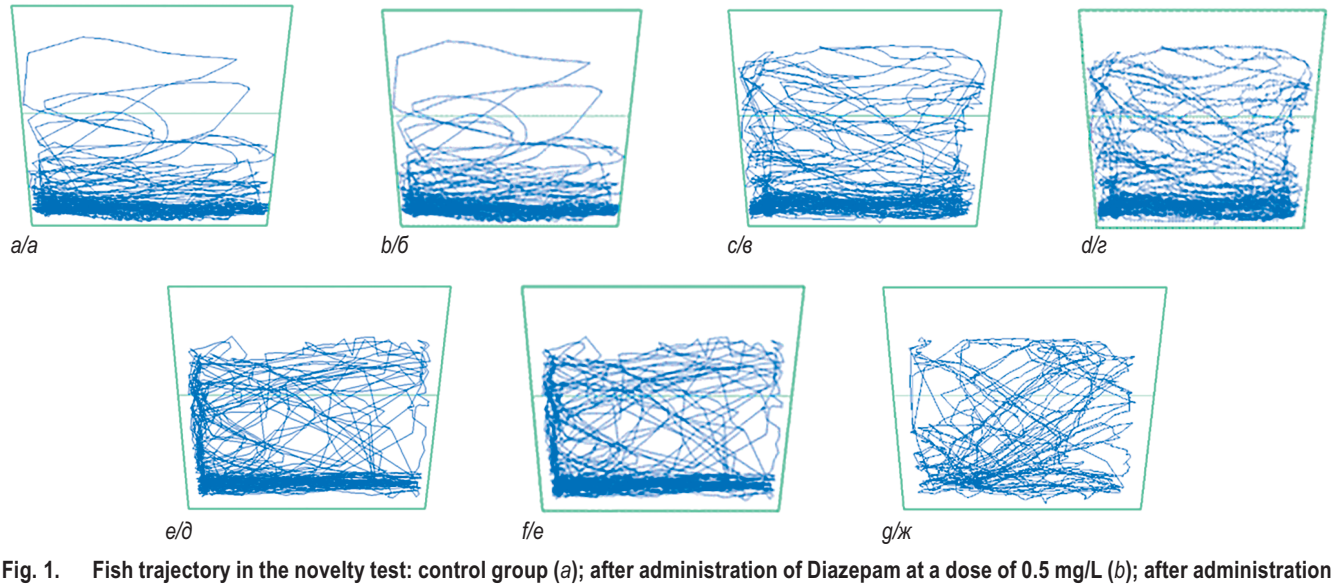

Fig. 1. Fish trajectory in the novelty test: control group (*a*); after administration of Diazepam at a dose of 0.5 mg/L (*b*); after administration of Diazepam at a dose of 1 mg/L (*c*); after administration of Tofisopam at a dose of 0.5 mg/L (*d*); after administration of Tofisopam at a dose of 1 mg/L (*e*); after administration of Hydroxyzine at a dose of 0.5 mg/L (*f*); after administration of Hydroxyzine at a dose of 1 mg/L (*g*)

Рис. 1. Траектория рыбы в тесте новизны у контрольной группы (*а*); применении Диазепама в дозе 0,5 мг/л (*б*); при применении Диазепама в дозе 1 мг/л (*в*); при применении Тофизопама в дозе 0,5 мг/л (*г*); при применении Тофизопама в дозе 1 мг/л (*д*); при применении Гидроксизина в дозе 0,5 мг/л (*е*); при применении Гидроксизина в дозе 1 мг/л (*ж*)

exhibited freezing (immobilization), and showed reduced motor activity. As a rule, the fish spent most of its time in the lower part of the observation tank, making small movements along it (Fig. 1). Along with this, freezing was often observed, the number and duration of which during the experiment were quite large, as was the time the fish spent in the lower part of the aquarium. The dynamics of the time the fish spent in the lower compartment remained largely unchanged, as did the dynamics of the time spent in the upper compartment and the number of transitions to the upper compartment. The obtained results are similar to those in literature sources. In particular, it was noted that the novel tank stress test is used in a number of laboratories to study anxiety levels in *Danio rerio* [21]. In this case, the fish, as a rule, swim in the lower part of the aquarium and often freeze (demonstrating freezing). Motor (and exploratory) behaviour is significantly reduced compared to behaviour in a home aquarium [16]. After a period of adaptation to the

observation aquarium, behaviour becomes much like that in a home aquarium, i.e., motor activity increases, freezing decreases, and the number of transitions to the upper half of the aquarium increases [12].

This research revealed several typical behavioural patterns in *Danio rerio* after administration of benzodiazepine tranquilizers Diazepam and Tofisopam. In this case, the fish swam not only in the lower part of the observation tank but also began to ascend to the upper area more frequently, particularly when Diazepam was administered at a dose of 1 mg/L (see Fig. 1, a). The number and duration of freezing episodes significantly decreased after Diazepam injection compared to the control group at doses of 0.5 and 1 mg/L. The duration time of the fish in the lower part of the aquarium after Diazepam injection significantly decreased, especially when using the drug in doses of 0.5 and 1 mg/L. The number of fish movements to the upper part of the observation aquarium after the administration of Diazepam significantly increased compared to the control group of animals, especially when using the drug at a dose of 1 mg/L. Under anxiolytic treatment, the path length of the fish did not change significantly in the range of doses used compared to the control group of animals, with the exception of the Hydroxyzine and Tofisopam group (Tables 1, 2). Similarly to the control group, freezing behaviour was observed. However, both the frequency and duration of freezing episodes significantly decreased compared to the control group ($p < 0.05$) (Table 1). The mean time spent by the fish in the bottom part of the tank also showed a significant decrease compared to the control animals ($p < 0.05$) (Table 2). Conversely, the number of entries into the upper section of the test aquarium during the trial was significantly increased compared to the control group ($p < 0.05$).

It has been previously shown that Phenazepam exerts a dose-dependent (0.1–1 mg/L) anxiolytic effect on novelty assessment in *Danio rerio*. The length of the fish trajectory after Fenazepam administration did not increase significantly compared to the control group of animals, in contrast to the effect of Diazepam (a significant increase in the trajectory of movement at doses of 0.5 and 1 mg/L). The number and duration of freezing episodes significantly decreased following Phenazepam administration compared to the control group. The time spent by the fish in the bottom of the tank was also significantly reduced after Phenazepam injection, particularly at a dose of 1 mg/L. The number of movements to the upper part of the observation aquarium after Phenazepam injection increased significantly compared to the control animals, particularly at a dose of 1 mg/L. Diazepam in contrast to Phenazepam causes increase in motor activity, which is not typical for tranquilizers. Our results align with literary data. It has been shown that Diazepam, unlike Phenazepam, is characterized by a dome-shaped dose-dependent effect [22]. Testing of anxiolytics in a number of laboratories to study the anti-anxiety effect in *Danio rerio* has demonstrated a fairly typical picture of behavioural patterns of fish, similar to the results we obtained. In this case, behavioural patterns under the influence of tranquilizers in *Danio rerio* are similar to those in rodents (rats, mice) — the most common object of study of tranquilizers. Literature describes anxiolytic effect of Diazepam [23], Chlordiazepoxide [21], and Phenazepam [24] in *Danio rerio*. It was noted that benzodiazepine anxiolytics Diazepam and Chlordiazepoxide are not inferior in the severity of the anti-anxiety effect to the 5HT1A receptor agonist Buspirone. The only difference in the action of benzodiazepine drugs, according to the authors, is the threshold anxiolytic dose to which animals respond without a sedative effect, that is, without a decrease in motor activity [21]. Moreover, in our studies, the characteristic of Diazepam's action was identified, which consists of relieving the stress of novelty with an increase in motor activity (atypical anxiolytic effect). This effect of the tranquilizer Diazepam may be explained by disinhibition due to activation of presynaptic GABA-A receptors [25]. When using Diazepam, the movements of fish were strictly confined to the bottom of the observation aquarium (see Fig. 1) compared to control animals, which indicates the depressant effect of the tranquilizer when its dose is increased. The obtained characteristics of Diazepam, undoubtedly, can be used in assessment of other classes (groups) of drugs. This supports the use of *Danio rerio* as an inexpensive but sufficiently informative test for pharmacological purposes. Thus, we can prove the effect of benzodiazepine tranquilizers on *Danio rerio* compared to the control group using both statistics and graphs.

Against the background of the action of anxiolytics, the average length of the fish's path did not change significantly in the range of doses used compared to the control group of animals, with the exception of the Hydroxyzine group (Table 2). Although, as in controls, freezing effect was observed. The number and time of fish freezing significantly decreased compared to the control group of animals ($p < 0.05$) (Table 2). The average time spent by fish in the lower part of the aquarium also decreased significantly compared to the control group of animals ($p < 0.05$) (Table 2). The number of times fish moved to the upper part of the test aquarium during the experiment increased significantly compared to the control group of animals ($p < 0.05$).

CONCLUSION

1. During the novelty test, *Danio rerio* spent most of their time in the lower section of the observation tank, making

small movements. Freezing episodes were frequently observed, with a high number and duration of freezing episodes during the experiment, as was the time spent in the lower section of the tank. The dynamics of the time spent in the lower and upper sections remained virtually unchanged.

2. The administration of benzodiazepine and non-benzodiazepine anxiolytics to *Danio rerio* produced a typical anxiolytic effect: increased exploration of the upper part of the aquarium, a decrease in the number and duration of freezing episodes, a reduction in the time spent by the fish in the lower part of the aquarium, and an increase in the number of entries into the upper part. At the same time, the total distance traveled (path length) did not change or even decreased after the administration of benzodiazepine tranquilizers (e.g., Tofisopam), whereas it increased following the administration of the non-benzodiazepine tranquilizer Hydroxyzine. The spatial pattern of movement also differed: non-benzodiazepine tranquilizers induced uniform swimming across almost all sectors of the aquarium, in contrast to the effect of benzodiazepine tranquilizers.

ADDITIONAL INFORMATION

Author contribution. Thereby, all authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study.

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Experiments with animals were carried out in accordance with international rules (Directive 2010/63/EU of the European Parliament and of the Council of the European Union of September 22, 2010 on the protection of animals used for scientific purposes).

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Вклад авторов. Все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией.

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