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Article

Interspecies Behavioral Variability of Medaka Fish Assessed by Comparative Phenomics

Gilbert Audira [1,2], Petrus Siregar [1,2], Kelvin H.-C. Chen [3], Marri Jmelou M. Roldan [4], Jong-Chin Huang [3 #], Hong-Thih Lai [5 #] and Chung-Der Hsiao [1,2,6,7 #]

1. Department of Chemistry, Chung Yuan Christian University, Chung-Li 320314, Taiwan
2. Department of Bioscience Technology, Chung Yuan Christian University, Chung-Li 320314, Taiwan
3. Department of Aquatic Biosciences, National Chiayi University, 300 University Rd. Chiayi, 600 Taiwan
4. Department of Applied Chemistry, National Pingtung University, Pingtung 900391, Taiwan
5. Faculty of Pharmacy and The Graduate School, University of Santo Tomas, Manila 1008, Philippines
6. Center for Nanotechnology, Chung Yuan Christian University, Chung-Li 320314, Taiwan
7. Research Center for Aquatic Toxicology and Pharmacology, Chung Yuan Christian University, Chung-Li 320314, Taiwan

Abstract: Recently, medaka has been used as a model organism in various research fields. However, even though it possesses several advantages over zebrafish, fewer studies were done in medaka than zebrafish, especially its behaviour. Thus, to provide more information regarding its behaviour and to demonstrate the behavioural differences between several species of medaka, we compared the behavioural performance and biomarker expression in the brain between four medaka fishes, which were *Oryzias latipes*, *O. dancena*, *O. woworae*, and *O. sinensis*. From the results, we found that each medaka species explicitly exhibited different behaviours to each other, which might be related to the different basal levels of several biomarkers. Furthermore, by phenomics and genomic-based clustering, the differences between these medaka fishes were further investigated. Interestingly, even though both phenomics and genomic-based clustering showed some resemblances to each other in terms of the interspecies relationship between medaka and zebrafish, however, in the medaka interspecies comparisons, this similarity was not displayed. Therefore, these results suggest a re-interpretation of several prior studies in comparative biology. We hope that these results contribute to the growing database of medaka fish phenotypes and provide one of the foundations for future phenomics studies of medaka fish.

Keywords: medaka; behavior; phenomics; interspecies

1. Introduction

Medaka is a small, oviparous freshwater teleost fish distributed in East Asia that is often found in rice fields and, therefore, has been called ‘ricefish’ [1]. Medaka, especially Japanese medaka (*Oryzias latipes*), has been used as a model organism in basic fish biology and behavioral studies. Furthermore, in recent years, the use of medaka as a model organism has highly contributed to knowledge in various research fields, such as genetics, toxicology, and behavior science [2]. Moreover, *O. latipes*

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has been proposed by the OECD as the standard fish for toxicology tests [3,4]. As an experimental organism, medaka possesses characteristics that are similar to those of the zebrafish, including its small size (adult 2-4 cm in length), fecundity, short generation time (2-3 months), simple dietary and habitat requirements, transparency of embryos, and availability of genomic information. Thus, it serves as a complementary model to the well-established zebrafish in many fields, including cancer research [1,5-7]. Also, medaka holds several edges over zebrafish, which come from its species-specific features. These features include hardiness, availability of highly polymorphic inbred strains, smaller genome size (800Mb), and the adaptation of photoperiod, higher salinity, and wide temperature (6-40 °C) [7]. These traits contribute to the vast usage of medaka as an aquatic toxicological model [5,6].

Nowadays, behavioral profiles of fish have become a widely used approach in toxicology and pharmacology, providing less cost, time, and space-consuming *in vivo* drug screening compared to traditional animal models. Despite their high sensitivity to various chemicals during their early developmental stages, fish also exhibit more complex behaviors, such as social interactions, learning, and memory, that may directly influence the survival of individuals and future population structures during juvenile and adult. These advantages may provide more options for aquatic neurotoxicity study [8,9]. It has recently become evident that medaka, especially *O. latipes*, possesses complex social and visually-evoked behaviors, such as aggressive behavior, predation, social learning, shoaling, mating preference, and startle response, emphasizing medaka as an emerging model for neurobehavioral research [5]. Moreover, their high genetic resemblance to humans established their role as a highly valuable human disorders study model. Additionally, it also affords many beneficial traits for behavioral studies [10]. These traits, including its central nervous system (CNS), are relatively simple, and its basic structure resembles those of amniotes. Thus, its genetic examination progresses rapidly, opening up new approaches for studying the genetic control of behavior [11-13]. Finally, yet importantly, medaka also can provide invaluable data for comparative research on zebrafish [14]. However, the current behavioral profiles based on this fish mainly focus on their early stages or only involve simple tests in aquatic toxicology research or ecotoxicological risk assessment [8]. Therefore, systemically comparing their behavioral features from simple to complex tasks is required to obtain full toxicity information of several toxicants.

Many animals exhibit interspecific behavioral diversities generated not only by genetic factors but also by learning and development. Interspecific behavioral diversities in some animal groups are thought to be a significant factor contributing to the emergence of social organization, influencing fitness, and are suggested to be under natural selection. Thus, in evolutionary ecology, this type of biological variation in behavioral characteristics has attracted the interest of many researchers. Interspecies variation is widely recognized in both clinical populations and animal models of human disorders. It is prevalent for the CNS phenotypes, such as neuropharmacological, behavioral, and toxic responses. However, the reasons causing individual differences maintained in natural populations and the connection between genetic polymorphisms and behavioral diversities remain unsolved. While interspecific behavioral diversities have been relatively well-studied in rodents and primates, aquatic models are far less characterized in this case. Initially, fish behavior was considered 'simple' and instinctive; however, it is recognized as homologous to mammals, complex, adaptive, context-specific, and highly variable nowadays [15,16].

Unfortunately, even though medaka is a small freshwater fish that is commonly used as an animal model for aquatic toxicology research, it has received relatively little attention in behavioral studies, specifically in interspecific behavioral diversities, compared to zebrafish [8,17]. Thus far, few reports describe differences in behavioral traits among medaka species and inbred strains, including a prior study by Hyodo-Taguchi that found some inbred strains of medaka tend to be attached to humans. In contrast, other inbred strains tend to avoid interactions with humans, and these responses may be mediated by a visual stimulus [18]. However, this difference has not been quantitatively analyzed based on paradigmatic experiments [15]. In addition, a prior study showed a great phenotypic diversity of *O. woworae* and studied the causative gene underlying divergence in sexually selected traits. Later, based on the red pectoral fins that are unique to the male of this fish, *csf1* was revealed as a causative gene for red pectoral fins that can contribute to male reproductive success by integration of genomic analysis and genome-editing technology. This result demonstrate that integrating genomic and phenomic approaches enables the identification of causative genes underlying selected traits [19]. In the present study, we examine the interspecific variation in behavioral responses in adult medaka fishes with a series of behavioral assays to determine whether these fishes can be used in behavioral studies in a way similar to how zebrafish are used or even in a more in-depth behavioral study, considering the toughness of medaka over zebrafish in several extreme environmental conditions. Furthermore, biochemical studies were also carried out to help in elucidating the behavior results. We hypothesized that each species has its species-specific features, and the results of this study may provide information for other researchers to decide which medaka fish is more suitable for the study of behavioral responses, with a particular focus on neuroscience, pharmacology, and toxicology. Additionally, the present result can contribute to the growing database of phenotypical differences between several medaka fish species. Moreover, comparative studies involving zebrafish and medaka are remarkably informative for identifying highly conserved genetic control mechanisms [20,21]. The overview of experimental design in the present study can be found in Figure A1.

2. Results

2.1. Novel tank assay performance comparison of four species of medaka fish

The novel tank assay is a test to inspect fish locomotor activity and its exploration ability to respond to the new environment [22]. Four behavioral endpoints to observe their locomotor activity, which were average speed, freezing, swimming, and rapid movement time ratios, were measured. Meanwhile, regarding the exploratory behavior, time in top duration, number of entries to the top, latency to enter the top, total distance traveled in the top, and average distance to the center of the tank (thigmotaxis) were quantified. During the test, each medaka group displayed a significantly different locomotor activity level to every other group. The highest level of locomotor activity was exhibited by *O. woworae* among the medaka groups. This phenomenon was shown by high average speed, swimming, rapid movement time ratios, and low level of freezing movement time ratio (Fig. 1A-D). However, *Danio rerio* still displayed higher locomotor activity than this medaka fish. Following *O. woworae*, a relatively high level of locomotor activity was also observed in *O. dancena*. Even though not as high as *O. woworae*, *O. dancena* also displayed a higher average speed and rapid movement ratio than the other two medaka fishes (Fig. 1A&D). Furthermore, a similar level of locomotor activity was detected in *O. latipes* and *O. sinensis*. While their average speed and rapid movement ratio were not statistically different from each other, different move types were observed in these fishes, supported by the low level of freezing time movement ratio and high level of swimming time movement ratio of *O. latipes* over *O. sinensis* (Fig. 1B-C). Next, regarding the exploratory behavior, a different response to the novel environment from the zebrafish was displayed by all medaka groups. These differences were supported by the significant difference of all exploratory behavior-related endpoints between zebrafish and all of the medaka fishes observed during the test (Fig. 1E-I). Interestingly, *O. sinensis* showed a longer time in top duration than other medaka fishes and even higher than zebrafish (Fig. 1E). However, a relatively short distance traveled in the top indicating that this medaka swam slower than the zebrafish in the top portion of the test tank, which was also supported with a low level of average speed displayed by this fish (Fig. 1A&I). Taken together, *O. woworae* possessed the highest locomotor activity among all of the medaka fishes, while zebrafish locomotor activity was still higher than that of medaka fish. Also, each medaka fish showed a specific and unique exploratory behavior, and this behavior was significantly different from the zebrafish. The detailed statistical analysis results of this test can be found in Table A1.

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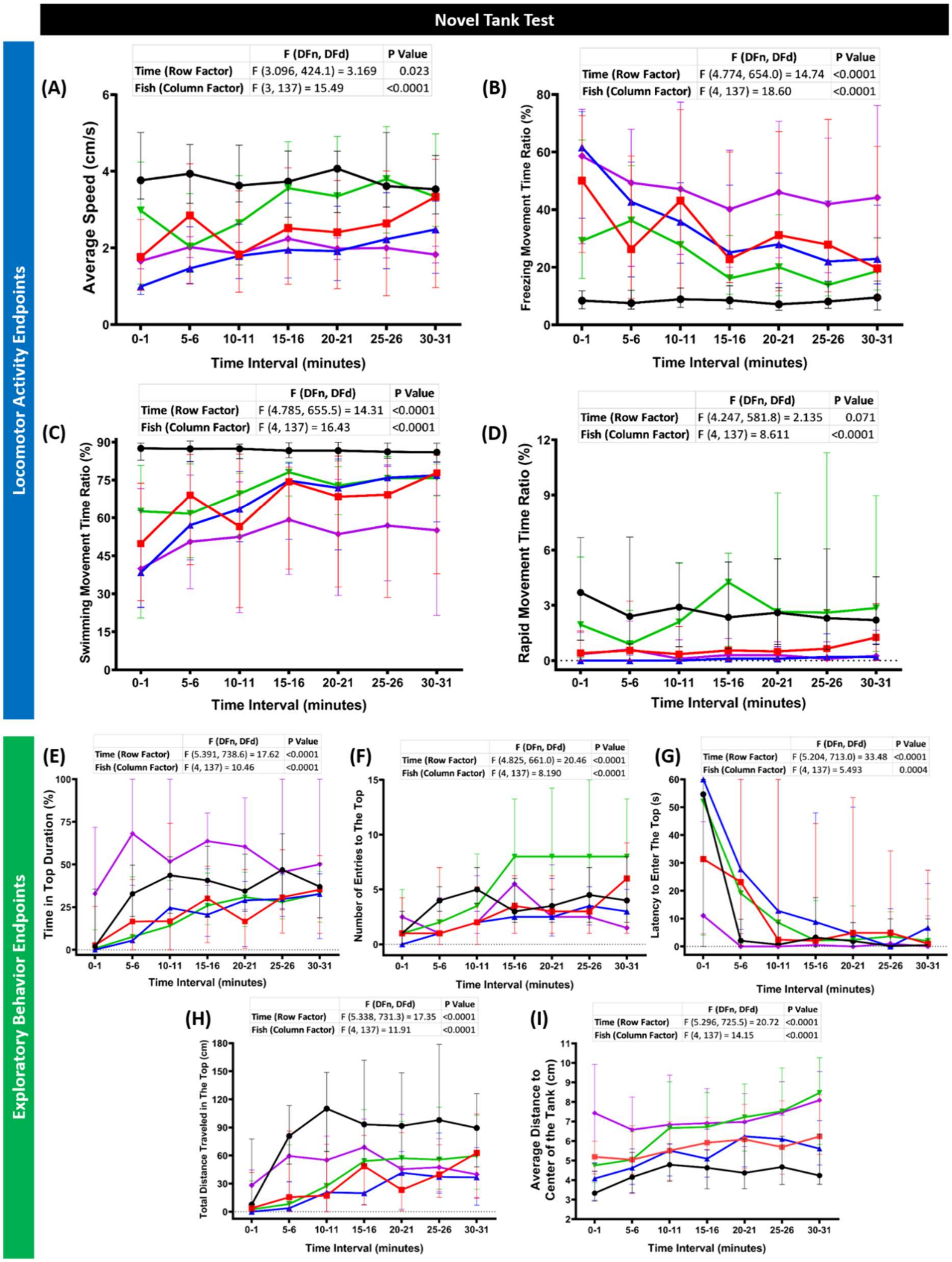


(A)

	F (DFn, DFd)	P Value
Time (Row Factor)	F (3.096, 424.1) = 3.169	0.023
Fish (Column Factor)	F (3, 137) = 15.49	<0.0001

(B)

	F (DFn, DFd)	P Value
Time (Row Factor)	F (4.774, 654.0) = 14.74	<0.0001
Fish (Column Factor)	F (4, 137) = 18.60	<0.0001

(C)

	F (DFn, DFd)	P Value
Time (Row Factor)	F (4.785, 655.5) = 14.31	<0.0001
Fish (Column Factor)	F (4, 137) = 16.43	<0.0001

(D)

	F (DFn, DFd)	P Value
Time (Row Factor)	F (4.247, 581.8) = 2.135	0.071
Fish (Column Factor)	F (4, 137) = 8.611	<0.0001

(E)

	F (DFn, DFd)	P Value
Time (Row Factor)	F (5.391, 738.6) = 17.62	<0.0001
Fish (Column Factor)	F (4, 137) = 10.46	<0.0001

(F)

	F (DFn, DFd)	P Value
Time (Row Factor)	F (4.825, 661.0) = 20.46	<0.0001
Fish (Column Factor)	F (4, 137) = 8.190	<0.0001

(G)

	F (DFn, DFd)	P Value
Time (Row Factor)	F (5.204, 713.0) = 33.48	<0.0001
Fish (Column Factor)	F (4, 137) = 5.493	0.0004

(H)

	F (DFn, DFd)	P Value
Time (Row Factor)	F (5.338, 731.3) = 17.35	<0.0001
Fish (Column Factor)	F (4, 137) = 11.91	<0.0001

(I)

	F (DFn, DFd)	P Value
Time (Row Factor)	F (5.296, 725.5) = 20.72	<0.0001
Fish (Column Factor)	F (4, 137) = 14.15	<0.0001

Figure 1. Novel tank behavior endpoints comparison between each medaka fish and AB strain zebrafish as the outgroup. (A) Average speed, (B) freezing time movement ratio, (C) swimming time movement ratio, (D) rapid movement time ratio, (E) time in top duration, (F) number of entries to the top, (G) latency to enter the top, (H) total distance traveled in the top, and (I) average distance to the center of the tank were analyzed. The data are expressed as the median with interquartile range (n = 30 for zebrafish, *O. dancena*, *O. latipes*, and *O. woworae*; n = 22 for *O. sinensis*).

2.2. *Aggressiveness comparison of four species of medaka fish*

Next, to evaluate the aggressiveness level of fishes, a mirror biting assay was carried out. This measurement was performed by counting the relative interact time of fish with its mirror-reflecting image [23]. Similar to the novel tank test result, each medaka fish displayed a different level of aggressiveness. The most pronounced aggressive behavior was shown by *O. sinensis*, followed by *O. woworae*. This finding was indicated by significantly higher mirror biting time percentage and longest duration in the mirror side percentage of these two medaka fishes than the other two medaka fishes, *O. dancena* and *O. latipes* (Fig. 2A&B). Interestingly, their level of aggressiveness was also found to be significantly higher than *Danio rerio*. On the other hand, *O. dancena* and *O. latipes* displayed a comparable level regarding their aggressiveness, which was also similar to *Danio rerio* (Fig. 2A&B). The detailed statistical analysis results of this test can be found in Table A2.

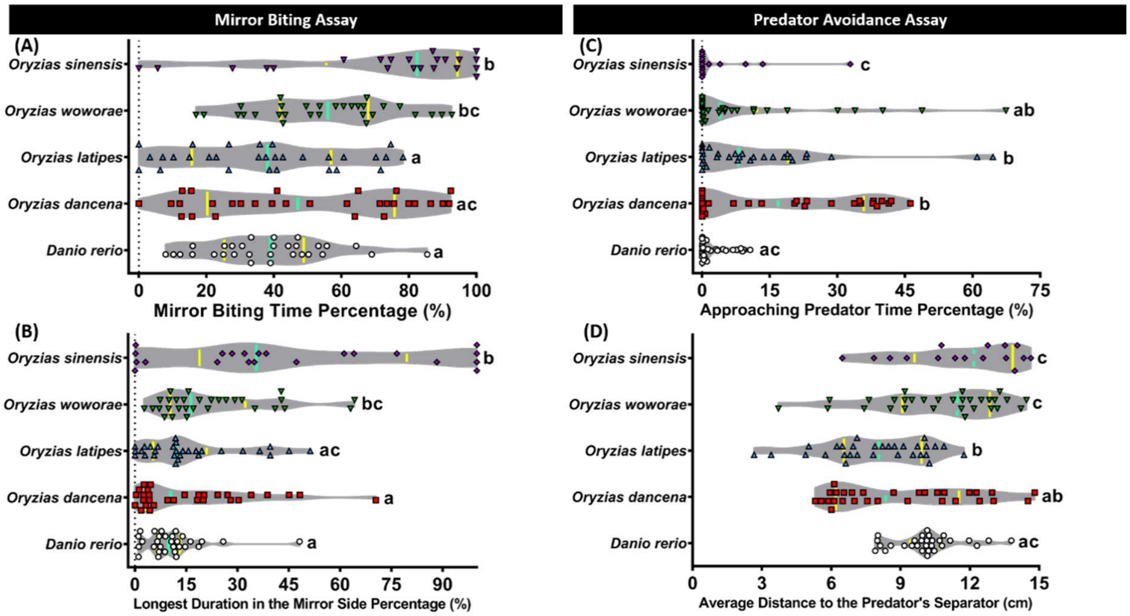


Figure 2. Mirror biting and predator avoidance behavior comparisons between medaka fish and AB strain zebrafish as the outgroup. (A) Mirror biting time percentage and (B) longest duration in the mirror side percentage were analyzed in the mirror biting assay (n = 30 for zebrafish, *O. dancena*, *O. latipes*, and *O. woworae*; n = 22 for *O. sinensis*). (C) Approaching predator time percentage and (D) average distance to the predator's separator were analyzed in the predator avoidance assay (n = 30 for zebrafish, *O. dancena*, *O. latipes*, and *O. woworae*; n = 16 for *O. sinensis*). The data were analyzed by the Kruskal-Wallis test continued with uncorrected Dunn's test. Different letters (a, b, c) on the error bars represent a significant difference ($p<0.05$). The violin plot's median and interquartile were labeled with the bold line colored with cyan and yellow.

2.3. *Comparison of predator avoidance test performance for four species of medaka fish*

Afterward, we evaluated the fish's fear level when facing their predators in the predator avoidance test [24]. The convict cichlid (*Amatitlania nigrofasciata*) was used in this study as a stimulus fish to promote the fear level of tested fish based on the previous protocol [25]. The least predator avoidance behavior was displayed by *O. dancena*, followed by *O. latipes*. A high level of approaching predator time percentage and a low level of the average distance to the predator's separator exhibited by these medaka fishes during the test indicated that they were not as fearful as the other fishes, including zebrafish (Fig. 2C&D). However, *O. woworae* and *O. sinensis* still displayed a quite clear predator avoidance behavior on a similar level with the zebrafish. The detailed statistical analysis results of this test can be found in Table A2.

2.4. Conspecific social interaction comparison of four species of medaka fish

Later, a conspecific social interaction test based on a similar rodent paradigm was conducted to evaluate the fish's social behavior. This test is conducted by observing their interactions with the conspecifics, and it is one of the useful assays to study fish social phenotypes [23]. From the results, *O. latipes* displayed the slightest interest to interact with their conspecific. This phenomenon was supported by a low level of conspecific interaction time percentage and the longest conspecific interaction percentage, and a high level of average distance to the conspecific separator (Fig. 3A-C). Following *O. latipes*, *O. dancena*, and *O. sinensis* showed a slightly more conspecific social interaction during the test. The most profound conspecific interaction among the tested medaka fishes was observed in the *O. woworae* group. In addition, this medaka fish also exhibited a comparable level of this social behavior with the *D. rerio*. The detailed statistical analysis results of this test can be found in Table A2.

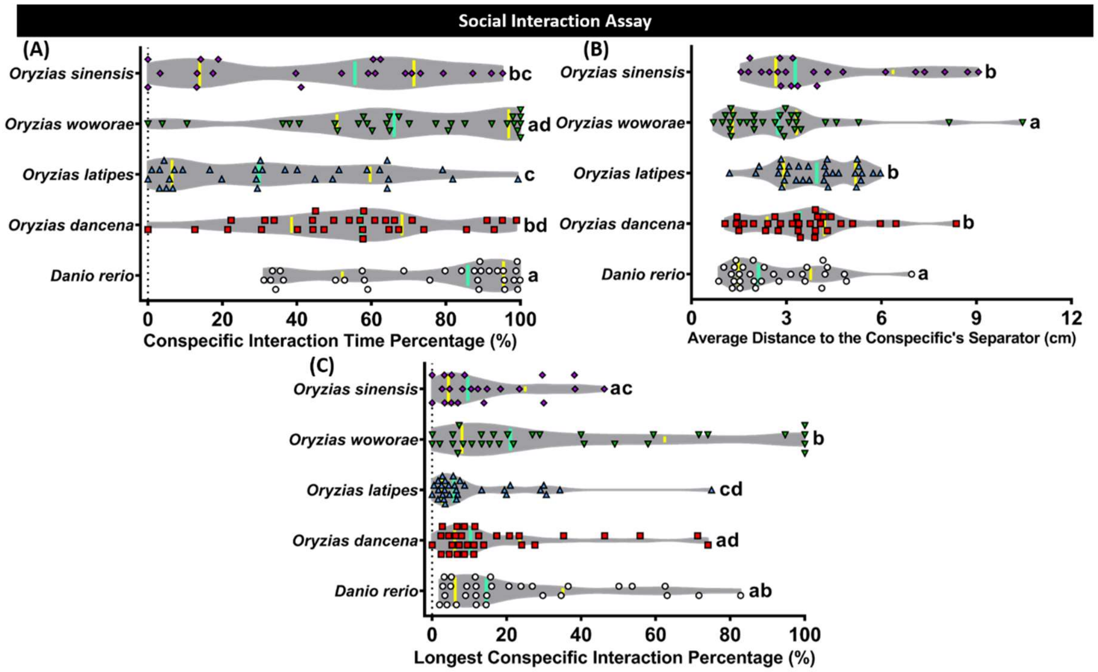


Figure 3. Conspecific social interaction behavior comparisons between medaka fish and AB strain zebrafish as the outgroup. (A) Conspecific interaction time percentage, (B) average distance to the conspecifics separator, and (C) longest conspecific interaction percentage were analyzed. The data were analyzed by the Kruskal-Wallis test continued with uncorrected Dunn's test. Different letters (a, b, c, d) on the error bars represent a significant difference ($p<0.05$) (n = 30 for zebrafish, *O. dancena*, *O. latipes*, and *O. woworae*; n = 21 for *O. sinensis*). The median and interquartile for the violin plot were labeled with the bold line colored with cyan and yellow.

2.5. Comparison of shoaling behavior for four species of medaka fish

Shoaling, an innate behavior for several fish to swim together in each medaka fish, was observed. Generally, this behavior was intended to reduce anxiety and the risk of being captured by the predators [26,27]. *O. latipes* and *O. dancena* formed a quite tight shoal during the test, shown by low levels of all behavioral endpoints, which are average inter-fish distance, average shoal area, average nearest neighbor distance, and average farthest neighbor distance (Fig. 4A-D). Meanwhile, *O. woworae* and *O. sinensis* displayed a slightly loose shoal, which is interesting since a similar shoal size was also observed in *D. rerio*. The detailed statistical analysis results of this test can be found in Table A2.

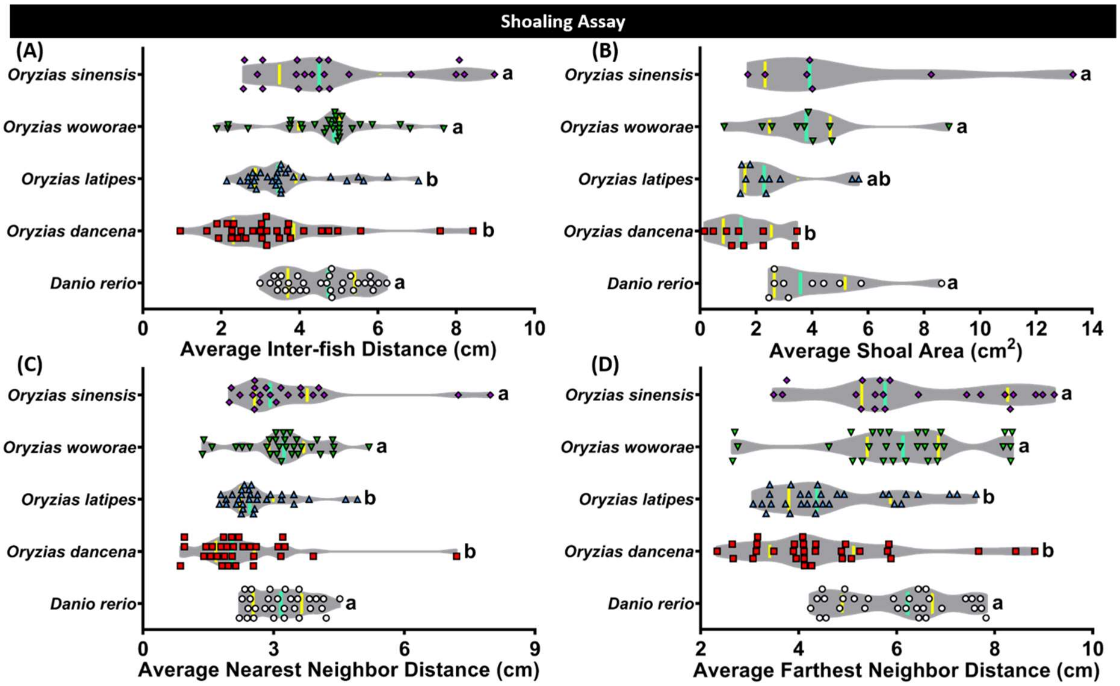
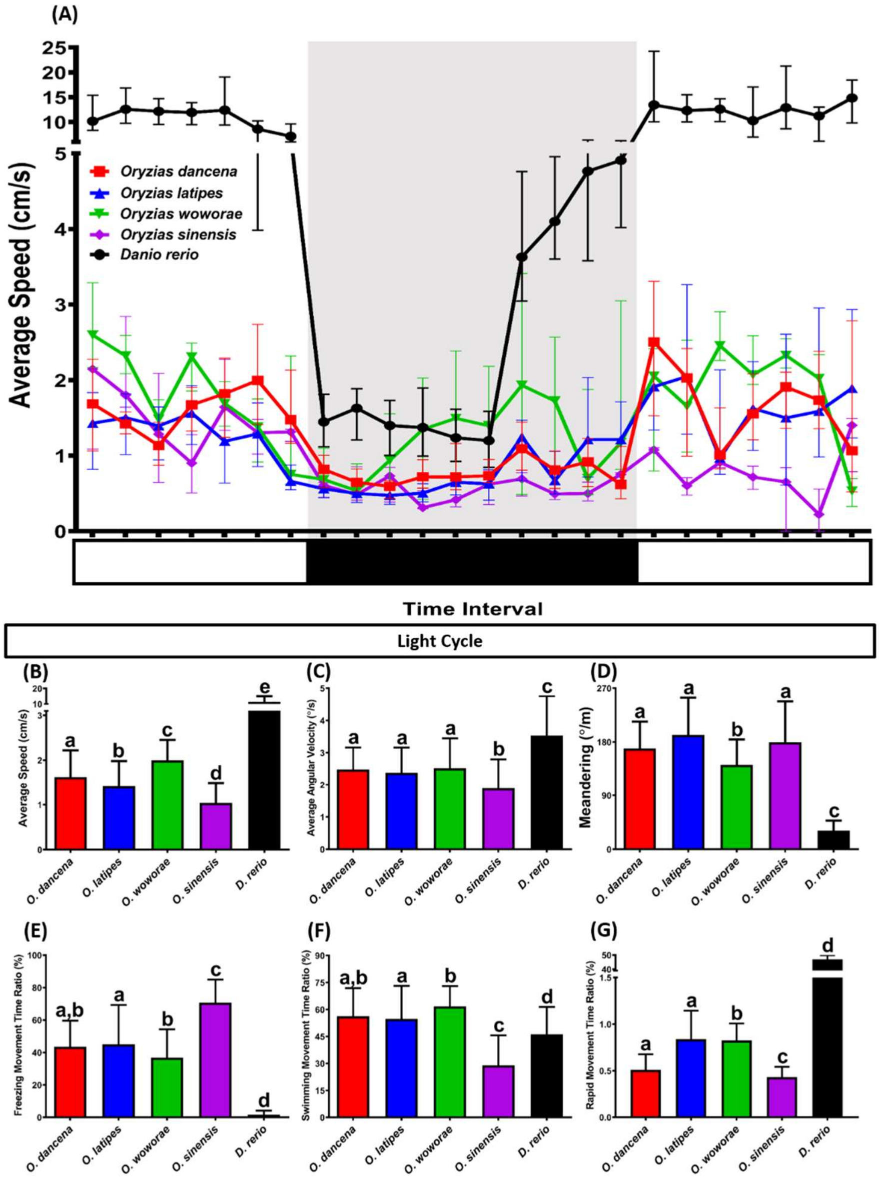


Figure 4. Shoaling behavior comparisons between medaka fish and AB strain zebrafish as the outgroup. (A) Average inter-fish distance, (B) average shoal area, (C) average nearest neighbor distance, and (D) average farthest neighbor distance were analyzed. Groups of three fish were tested for shoaling behavior. The data were analyzed by the Kruskal-Wallis test continued with uncorrected Dunn's test. Different letters (a, b) on the error bars represent a significant difference ($p<0.05$) (n = 30 for zebrafish, *O. dancena*, *O. latipes*, and *O. woworae*; n = 21 for *O. sinensis*). The median and interquartile for the violin plot were labeled with the bold line colored with cyan and yellow.

2.6. Comparison of circadian locomotor activity rhythms for four medaka species

Next, we assessed their circadian locomotor activity rhythm since this daily rhythm of gross locomotor activity is frequently used as an assay of circadian rhythmicity of animals, especially mammals [28]. From the results, all medaka fishes displayed a significantly different circadian locomotor activity pattern compared to the zebrafish (Fig. 5A). The high levels of locomotor activity showed these differences during both day and night cycles. Meanwhile, regarding the medaka fish results, differences between each species were also observed during the experiment. In the day cycle, the highest locomotor activity was exhibited by *O. sinensis*, followed by *O. dancena*, *O. latipes*, and *O. sinensis*, which was consistent with the novel tank test results, shown by the differences in average speed, average angular velocity, freezing, swimming, and rapid movement time ratios (Fig. 5B-C, D-G). Also, in terms of movement orientation, more pronounced zig zag-like movement was shown by every medaka fish compared to zebrafish. This phenomenon was shown by the high level of meandering measured during the day cycle, which was also observed during the night cycle (Fig. 5, J). Furthermore, similar results regarding locomotor activity were also found in the night cycle, where *O. woworae* possessed the highest locomotor activity among the rest of the medaka fishes, indicated by the differences in average speed, average angular velocity, freezing, swimming, and rapid movement time ratios (Fig. 5H-I, K-M). The detailed statistical analysis results of this test can be found in Table A2.



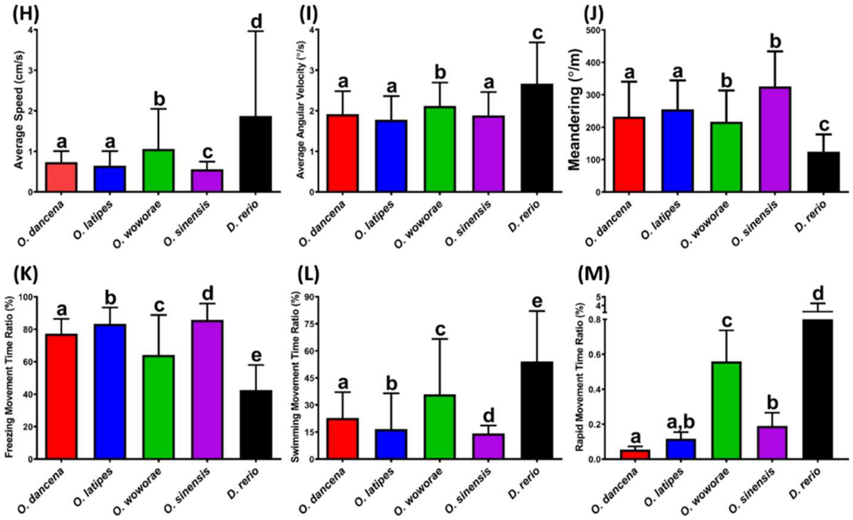


Figure 5. The circadian locomotor activity rhythm of medaka fish and AB strain zebrafish as the outgroup (A) Comparison of the average speed between fishes during the day and night cycles. Comparisons of (B, H) average speed, (C, I) average angular velocity, (D, J) meandering, (E, K) freezing movement time ratio, (F, L) swimming movement time ratio, and (G, M) rapid movement time ratio in the day and night cycles, respectively. Data were presented as median with interquartile range, except for G and M, which were presented as mean with a 95% confidence interval (CI). Data were analyzed by Kruskal-Wallis test continued with uncorrected Dunn's test. Different letters (a, b, c, d, e) on the error bars represent a significant difference ($p<0.05$) (n = 18 for *O. dancena*, *O. latipes*, *O. wowora*, and *D. rerio*; n = 12 for *O. sinensis*).

2.7. *Biochemical Assay of Biomarker Expression in the Brain*

Since behavior variations between each medaka fish were observed in previous behavioral tests, it was intriguing to investigate the contents of several neurotransmitters, antioxidant activity, and oxidative stress that might play roles in these differences. Interestingly, based on the neurotransmitter levels, we found that *O. wowora* and *O. dancena* showed significantly higher levels of these neurotransmitters, except for AChE, than *O. latipes* and *O. sinensis*. Furthermore, similar results were also observed in stress hormones, which is cortisol (Table 1). On the other hand, while *O. wowora* still showed the highest level of AChE, *O. dancena* possessed a comparable level of this neurotransmitter to O sinensis followed by *O. latipes* that also displayed a similar level of AChE to *O. sinensis*. In addition, this pattern was also found in the oxidative stress-related markers, which are CAT and ROS (Table 1). The detailed statistical analysis results of this test can be found in Table A3.

Biomarkers	***O. dancena***		***O. latipes***		***O. woworae***		***O. sinensis***		**Unit**
	Concentration	SSD* letter	Concentration	SSD letter	Concentration	SSD letter	Concentration	SSD letter	
5-HT	36.21 ± 11.25	**a**	25.95 ± 5.634	**b**	40.59 ± 7.444	**a**	27.16 ± 7.525	**b**	ng/total protein (mg)
CAT	15.83 ± 5.214	**ac**	10.16 ± 2.194	**b**	14.35 ± 2.980	**a**	10.77 ± 2.829	**bc**	ng/total protein (mg)
NE	1.512 ± 0.6271	**a**	0.8307 ± 0.2756	**b**	1.653 ± 0.5085	**a**	1.011 ± 0.4499	**b**	ng/total protein (mg)
DA	22.86 ± 9.282	**a**	12.84 ± 3.738	**b**	24.93 ± 5.888	**a**	14.45 ± 4.942	**b**	pg/total protein (mg)
Cortisol	228.1 ± 69.08	**a**	135.5 ± 44.60	**b**	283.3 ± 68.17	**a**	146.9 ± 68.29	**b**	pg/total protein (mg)
AChE	29.82 ± 6.940	**ac**	17.09 ± 9.654	**b**	35.92 ± 7.873	**a**	23.44 ± 15.40	**bc**	U/total protein (mg)
ROS	133.5 ± 44.48	**ac**	80.81 ± 31.65	**b**	199 ± 69.21	**a**	89.78 ± 44.00	**bc**	IU/total protein (mg)

Table 1. Comparison of neurotransmitters, antioxidant activity, and oxidative stress contents in medaka brain tissues measured using enzyme-linked immunosorbent assay (ELISA). The data are expressed as the mean with SD. Kruskal-Wallis test continued with Uncorrected Dunn's test was used to analyze the data. Different SSD letters (a, b, c) represent significant differences ($p < 0.05$; $n = 10$ for all groups; *SSD = Statistically Significant Difference).

2.8. PCA analysis and hierarchical clustering analysis of several medaka fish behavioral endpoints

To explore behavioral phenomics between several different medaka fish species, principal component analysis (PCA), hierarchical clustering, and heatmap comparison were performed after all the behavioral tests. This process is also vital to reduce the data dimension and complexity. As the outgroup, AB strain zebrafish (*D. rerio*) behavioral data were included to conduct a more profound study about the behavioral pattern differences between tested medaka fishes. Two major clusters were generated from the hierarchical clustering result, separating *O. latipes*, *O. dancena*, and *O. sinensis* in one cluster with *O. woworae* and *D. rerio* in another cluster (Fig. 6A&B). In the first cluster, *O. latipes* and *O. dancena* were closer to each other than *O. sinensis*. This categorization was plausible since these medaka fishes exhibited several behaviors that were not observed in other fish, such as less pronounced predator avoidance behavior (endpoint 3-1) and tightened shoal formed (endpoint 5-1 to 5-4). Meanwhile, even though *O. sinensis* was found to be in this cluster, it showed a different behavioral pattern to the two medaka fishes in several vital endpoints. The most evident difference was in one of the novel tank test endpoints, a time in top duration (endpoint 1-1-5 and 1-2-5). As already mentioned in Fig. 1, a significantly high level of this endpoint was displayed by this medaka fish during the whole 30 minutes of the novel tank test. Next, it was found that *O. woworae* and *D. rerio* belonged in the same cluster. After further investigation, we discovered that these two fishes exhibited similar behavior patterns, especially in the locomotor activity-related endpoints, such as average speed and rapid movement ratio. A strong conspecific interaction shown by these fishes also becomes one of the factors that defined this grouping. The definition of all of the behavioral endpoints was described in Supplementary Table A4.

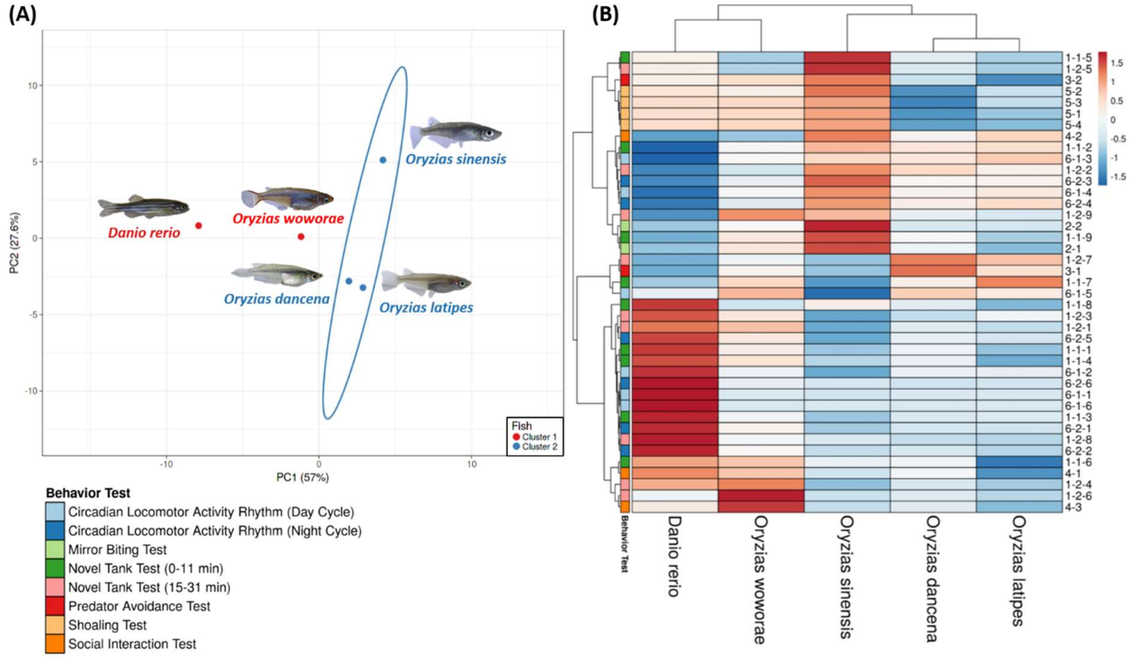


Figure 6. (A) Principal Component Analysis and (B) hierarchical clustering analysis of multiple behavior endpoints in several different medaka species zebrafish. In Fig. 6A, two major clusters from hierarchical clustering analysis results were marked with the red color (1st cluster) and blue (2nd cluster) circle. The behavioral data from another fish species (*Danio rerio*) was also included as the outgroup to conduct a more in-depth study about their behavior differences pattern.

2.9. Phylogenetic relationships of four medaka species

To verify the relationship between each medaka fish species tested, we constructed a phylogenetic tree of medaka and *D. rerio* as the outgroup based on the NADH dehydrogenase subunit two gene (ND2). The medaka fish were classified into three groups from the phylogenetic tree, including celebensis, javanicus, and latipes species groups, as described by Murata et al. [29]. Interestingly, this genetic-based grouping follows with the salinity tolerances of these fishes, which are euryhaline for *O. dancena*, *O. latipes*, and *O. sinensis* [30-32] and stenohaline for *O. woworae* and *D. rerio* [33,34].

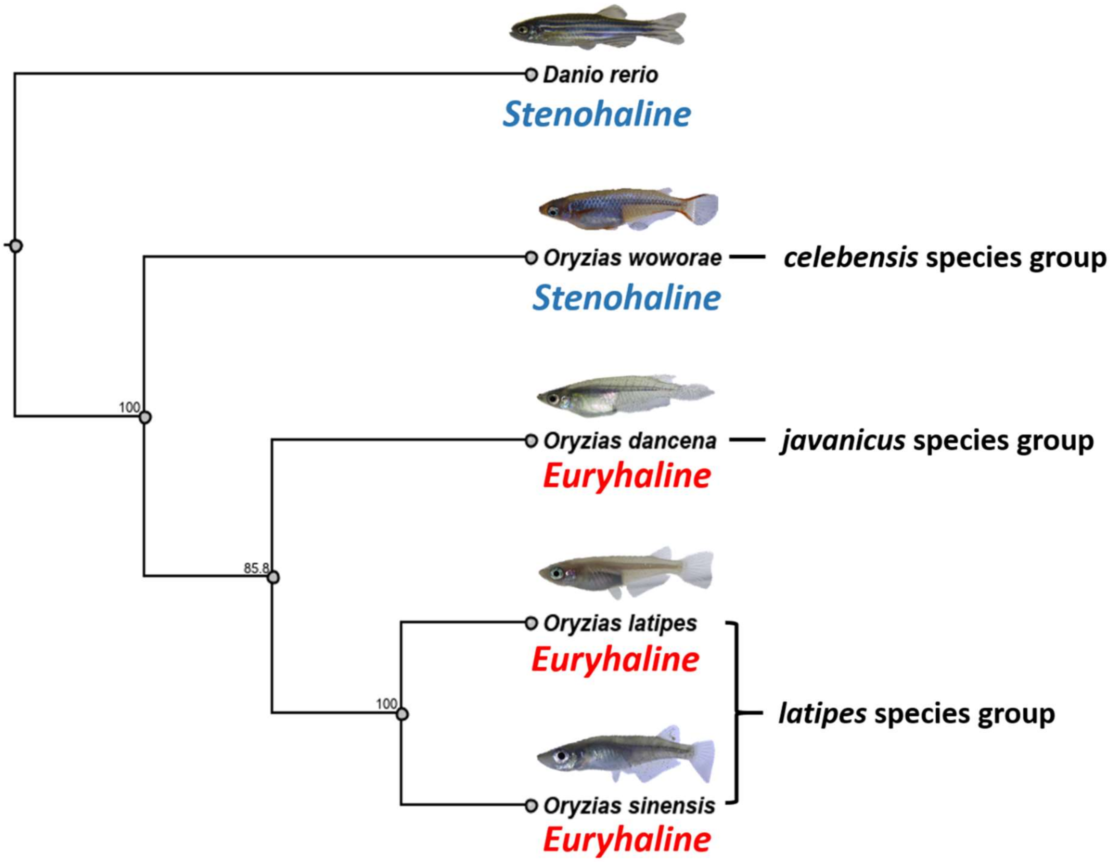


Figure 7. Phylogenetic relationships among four *Oryzias* species and *Danio rerio* inferred from the datasets of NADH dehydrogenase subunit 2 gene (ND2). The phylogenetic relationships were analyzed by the maximum-likelihood method. The numbers beside the branches indicate the bootstrap values. Based on the prior publication by Murata et al. (2019), *O. latipes* and *O. sinensis* genetically belong to the latipes species group while *O. dancena* and *O. woworae* belong to javanicus and celebensis species groups, respectively. Previous studies revealed that *O. dancena*, *O. latipes*, and *O. sinensis* exhibited euryhalinity, whereas *O. woworae* and *D. rerio* exhibited stenohalinity.

3. Discussion

This is the first study to demonstrate the comparison of innate behaviors between several medaka fish species to the best of our knowledge. As we predicted, each species has its species-specific features in each behavior tested. *O. woworae* displayed the highest locomotor activity, while each medaka exhibited a unique exploratory behavior to each other. Moreover, this phenomenon was also obviously shown in the circadian locomotor activity rhythm test. Furthermore, this fish, together with *O. sinensis*, exhibited a significantly high aggressiveness level than *D. rerio*. Meanwhile, regarding predator avoidance behavior, these fishes showed similar fear-like behavior to *D. rerio*, while this behavior response to the predator was not clearly observed in *O. dancena* and *O. latipes*. *O. woworae* also showed a more distinguished social behavior in the same manner with *D. rerio* than other medaka fishes. Lastly, while all medaka fishes showed interest in forming a shoal with their conspecifics, a quite tight shoal was displayed by *O. dancena* and *O. latipes*.

The novel tank test has been widely used to study animal habituation. Generally, habituation is defined as a change in locomotor activity and exploratory behavior over time to sustain an animal's survivability [35]. Based on the prior study, this behavior test on zebrafish provided results comparable to other similar studies in other animal species, including medaka [36]. The typical behavioral patterns of all medaka changes in a stereotypical manner were demonstrated from the current results. This phenomenon indicated that animals become familiar with the novel location as the exposure time increased. Consistent with this result, a previous study demonstrated a similar pattern change during the habituation of *O. latipes* in open-field test [14]. In terms of their differences from zebrafish, all medaka fishes exhibited a relatively lower locomotor activity than zebrafish most of the time. This result is somewhat similar to a prior study in another species of medaka (*O. javanicus*), which found that the medaka fish were passive compared to *D. rerio* that were more active and aggressive [37]. Moreover, a high thigmotaxis level, a preference of animals towards the periphery of a novel arena and avoiding the center area, during the test was displayed by all medaka fish [38]. This result is plausible since zebrafish tend to spend time in the center area of a tank after it is habituated, while this behavior is not shown in medaka [39]. All medaka fishes displayed similar phases of habituation in the novel environment. This finding confirmed the usefulness of medaka and novel tank tests to investigate habituation phenotype even though each species possessed different degrees of locomotor activity and exploratory behavior.

Similar to zebrafish, mirror approaching behavior in medaka is defined as a social behavior model [40]. When a single medaka is placed in a tank, it swims freely in all directions; however, the same individual will tend to swim close to the mirror when a mirror is placed on one side of the tank [41]. Unfortunately, while this paradigm is well studied in medaka, most of the studies were done in *O. latipes* [5,41]. Thus, this is the first study that revealed the mirror approaching behavior in other medaka species. Surprisingly, in this study, while *O. latipes* displayed a similar mirror approaching behavior to *D. rerio*, other medaka fishes exhibited a more robust mirror approaching behaviors. Since mirror approaching behavior in medaka is considered a simple and robust model of socially induced anxiety, the high level of mirror biting time might indicate a higher stress level in these fishes than *O. latipes* and *D. rerio*. Interestingly, cortisol, one of the primary fish hormones, was found to be lower in *O. latipes* compared to other medaka fishes, especially *O. dancena* and *O. latipes*. These observations indicate that cortisol might affect the differences in their aggressive behavior since it is involved in medaka fish's stress response [42]. Also, this speculation was based on the prior study in diazepam and fluoxetine, which are anxiolytic agents that decreased the mirror biting time and the stress response measured by cortisol level [5,41,43]. Furthermore, different basal levels of ROS, molecules responsible for signaling stress response, could also influence the differences in their behavior. This possibility was based on the previous research in adult zebrafish that showed anxiety-like or stress-like behavior, which likely contributed by higher ROS levels [44,45]. This speculation is also supported by the variation in catalase's basal levels of each medaka fish. Catalase is primarily a peroxisomal enzyme that catalyzes the enzymatic decomposition of H_2O_2, and in rats, it is associated with depression-like behavior associated with Alzheimer's disease improvement [46,47]. Lastly, aggression also has been linked to serotonergic function in a variety of invertebrate and vertebrate species. Generally, a high level of serotonin activity clearly shown in the results is associated with low levels of aggressive behavior, [48].

Avoiding predation is an essential behavioral reaction critical for survival and may have a significant fitness component. Many animals, including fish, sense a danger of predation through a multitude of methods, such as alarm signals, calls, and chemical cues. To avoid predation, the animals have to respond to stimuli that represent the presence of danger properly [24,49]. It not only helps the researchers to elucidate the evolution and ecology of the studied species, but analysis of such fear responses may have clinical relevance [50]. Unfortunately, regardless of the promising advantages, only a few studies of this predation response in medaka. Moreover, most of these studies only used *O. latipes* as an animal model [49,51]. However, in the current study, we found that each medaka species responded to the presence of *A. nigrofasciata* as the fear stimulus in different magnitudes. Interestingly, while *O. latipes* fear responses were clearly demonstrated in a prior study, this behavior was not shown in this experiment. This difference might be due to the different stimuli used to elicit the alarm response. While we stimulated the fear response by exploiting the visual cue from another fish, the prior study used a conspecific skin extract as the chemical cue of alarm substance [49]. Thus, each medaka fish might respond differently to other fish species or a different stimulus upon their first exposure to these fish, which need to be confirmed in future studies. In addition, a more robust reaction in response to the predator stimulus was observed in *O. woworae* and *O. sinensis*. These results might indicate that these medaka fishes displayed a fear response in the presence of the stimulus since their response was similar to *D. rerio* that is already well-studied and proven to elicit a fear response in a similar situation [25,52,53]. One possibility that caused the differences is related to each specific basal serotonin content of each medaka fish. As mentioned above, serotonin is a good physiological indicator of various types of stress in fish. Mosienko *et al*. demonstrated serotonin deficiency in certain regions of the brain exhibited by Tph2-knockout medaka was associated with elevated stress and fear-related behaviors [54].

Generally, the development of social behavior is observed among various vertebrates from fish to mammals and coordinated with social factors, such as the presence of conspecifics, physiologic, and environmental factors [55,56]. In medaka fish, especially *O. latipes*, this social behavior had been well addressed previously. A prior study discovered that medaka fish robustly and reproducibly maintained proximity to a single target conspecific fish since it is attracted by conspecifics' biological motion [57,58]. However, whether other medaka species also possess this social behavior by the presence of conspecific remains unknown. Even though *O. latipes* was found to maintain proximity to its conspecific, other medaka species also displayed similar behavior, even in a higher magnitude and more robust manner. These results are intriguing since some of these medaka species, especially *O. woworae*, displayed social behavior as clear as *D. rerio*, a well-established animal model for this behavior [25,59]. Furthermore, shoaling, a simple form of affective behavior displayed in social fish species, is usually observed in small fish, including medaka (*O. latipes*) and zebrafish. This behavior may directly influence the survival of individuals and future population structures [9]. In the previous study, medaka was demonstrated to form shoals only with conspecifics and not with other species [60]. In this study, all medaka fishes formed a shoal that even tightened than *D. rerio* for some medaka species, which were *O. dancena* and *O. latipes*. These results are plausible since medaka are known to have high visual acuity and exhibit a strong tendency to form shoals [61]. The differences in the social behaviors of each medaka fish might be related to the differences in their dopamine and norepinephrine levels. These neurotransmitters are involved in the organization of stress response in vertebrates, and together with serotonin, they are involved in the mediation of behavioral and neuroendocrine responses to social stress [62,63]. A prior study in teleost shows that the brain dopamine level increased after being stressed. Thus, the current results indicate that some medaka fishes might have different levels of stress compared to the others, which is consistent with the mirror biting test results. The stress is plausible since recent research discovered that grouping and pairing are more stressful to male medaka than isolation [42]. Regarding norepinephrine content, the previous finding found that juvenile lake sturgeon (*Acipenser fulvescens*) held in isolation had a significantly longer norepinephrine response than fish held with conspecifics [64]. In addition, another study in zebrafish found that whole-brain dopamine level was also found to be associated with the development of shoaling [65]. In a prior study, dopamine level was found to have rapidly risen in the brain of adult zebrafish in response to social stimuli [66].

The time of oviposition in medaka fish, especially in *O. latipes*, was well accounted a many years ago. Interestingly, this rhythm is controlled by a circadian pacemaker entrained by LD cycles since it was gradually disappeared under continuous light [67]. The locomotor activity rhythm seems to be dependent on the light intensity during dark periods [68]. Several gene expressions, including *per*, *cry*, *clock*, and *bmal*, are some of the key elements of the medaka clock mechanism. The differential maturation of these elements depends on the presence of the chorion and the developmental stage. Here, typical diurnal circadian locomotor activity rhythms were displayed by all of the medaka fishes. Even though they were not as pronounced as in *D. rerio*, they are consistent with their natural behaviors [69]. This phenomenon might be explained by the circadian rhythm trait of medaka fish, especially *O. latipes*, which can be entrained by feeding time [70]. Thus, the feeding schedule that occurred during the day cycle in the present study might play a role in affecting this behavior since a prior study demonstrated that this entrainable rhythm is shown to be a persistent behavior. In their study, it was discovered that after a three-day fast, a meal-feeding readily entrained circadian rhythm of agonistic behavior of *O. latipes* was remained fixed to the feeding time [71]. However, the reader should not conclude that all medaka species are capable of being entrained to feeding. Thus, future studies are needed to be conducted to verify this speculation. Furthermore, *O. woworae* was found to be the most active fish regarding their locomotor activity levels, compared to other medaka fishes, which is consistent with the novel tank test results. This phenomenon could be related to the high level of serotonin and AChE measured in the fish. This speculation was taken since serotonin has been reported to play a positive role in regulating locomotor activity in many animals, while AChE is an enzyme responsible for the breakdown of acetylcholine, which plays an important role in modulating neuromuscular activity in neural synapses [72]. This trend is also observed in other medaka fishes.

In summary, the behavioral differences between each medaka fish might be associated with their different basal level of neurotransmitters. This phenomenon has been briefly noted in a previous study. However, the comparison only covered three kingdoms of living organisms [73]. Nevertheless, even though this difference was not fully investigated, it has been mentioned in several prior studies, including in fish. In normoxia conditions, three fish species, goldfish, tilapia, and carp, possessed different energy status levels, lactic acid accumulation, and amino acid patterns in brain tissue [74]. In addition, by using a combination of immunohistochemistry and confocal imaging, a previous experiment confirmed the differences in serotonin and acetylcholine contents in the gill's filament and lamellae of various fish species, including goldfish (*Carasius auratus*), sockeye salmon (*Oncorhynchus nerka*), trout, and *O. latipes* [75]. Furthermore, several prior findings also found some variations in the levels of adrenaline and noradrenaline stored within the chromaffin tissue of various species of fish, such as cyclostomes, dipnoans, elasmobranchs, ganoids, and teleosts [76]. Lastly, Aprison *et al*. also found some differences regarding glycine concentration, an amino acid that has potent inhibitory actions on the vertebrate nervous system, at various levels of the neuraxis in five different vertebrates [77]. Additionally, these phenomena might also related to the different genes underlying divergences in selected traits between species, particularly in vertebrates, which need further studies to confirm this hypothesis [78]. Overall, this is the first study comparing the basal levels of neurotransmitters between each species of medaka fishes to the best of our knowledge. Thus, further studies are required to deeply investigate this phenomenon.

Later, to precisely determine the phylogenetic position of each medaka fishes and *D. rerio*, we estimated the phylogenetic relationship using published whole-genome datasets as references. The tree indicates that *O. sinensis*, *O. latipes*, and *O. dancena* formed a monophyletic group, which is consistent with previous trees based on a concatenated mitochondrial sequence matrix [79]. This result is plausible since *O. sinensis* is described as the subspecies *O. latipes sinensis* Chen. These species have approximately the same length of anal-fin rays and chromosome arms numbering 58 or more, distinguishing them from putative close relatives [32,80]. Furthermore, *O. dancena* and *O. woworae* were

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phylogenetically clearly separated from *O. sinensis* and *O. latipes*, which is also in agreement with other phylogenetic trees from previous studies that are based on the nuclear *tyrosinase*, mitochondrial 12S, and 16s rRNA genes, and several characters. Based on these studies, the *Oryzias* species itself is ramified into three monophyletic groups. While *O. dancena* belongs to the *javanicus* clade, both *O. sinensis* and *O. latipes* belong to the *latipes* clade, which can elucidate the phylogenetic tree result of the present study. The *javanicus* clade is a monoarmed chromosome group and possesses subtelo- and acrocentric chromosomes that distinguish it from other clades [6,32,79,81]. Next, as reported in a phylogeny based on the 3440-bp concatenated mitochondrial and nuclear sequences by Mokodongan and Yamahira, *O. woworae* was genetically distinct from other medaka fishes, such as *O. latipes* and *O. javanicus*. This grouping might also cause by the different wider salinity tolerance between the medaka fishes. While medaka fishes, including *O. latipes*, *O. sinensis*, and *O. dancena*, have a wide salinity tolerance, this trait was not possessed by *O. woworae*, which might also explain the close distance between this fish and *D. rerio* in the phylogenetic tree. However, based on the preliminary phylogenomic analyses, the distance between zebrafish and medaka is due to the differences in the 20 nuclear protein-coding genes [82-85]. Interestingly, the phylogenetic tree and phenomics-based hierarchical cluster generated in this study displayed several resemblances, including the interspecies relationship between zebrafish and the medaka fishes. However, a slightly different result to the phylogenetic tree was observed in the medaka interspecies comparisons. The phenomic-based hierarchical clustering result showed that *O. latipes* and *O. dancena* appeared to be more closely related to each other than either of them was to *O. sinensis*. Thus, this is evidence that genetic-based phylogenetic analyses might have a different relationship among the species to the phenomics-based cluster analysis. However, a prior study has already addressed those differences regarding the relationship between zebrafish, medaka, pufferfish, and cichlids. In their study, the molecular data supported a close relationship between atherinomorphs (including the medaka and platy) and putatively more derived perform fish such as the cichlids. At the same time, previously, the atherinomorpha have been historically placed in an intermediate position among the other branches of the acanthomorph tree since they share several putative 'primitive' morphological features with more basal teleosts. Thus, their phylogenomic analysis revealed an unexpected relationship among the other three species, contrary to traditionally held systematic views based on morphology [85].

Next, to observe their reproducibility, we also calculated the coefficient of variation of each medaka fish species and *D. rerio* from every behavioral test. Based on the result in Table A5, *O. woworae* displayed the lowest average coefficient of variation between all medaka fishes even though it was still higher than *D. rerio*. This result indicates the stronger reproducibility of *O. woworae* than other medaka fish in these behavior tests. A high coefficient of variation may impact the animal usage of an experiment since this condition is a problem to obtain significant results. Thus, high sample size is become mandatory to overcome this problem. Moreover, this problem also means that the researchers might face ethical-related issues [17,86].

4. Materials and Methods

4.1. Animal Husbandry

Indian ricefish (*Oryzias dancena*) and Daisy's ricefish (*Oryzias woworae*) were purchased from a local pet store, while Chinese ricefish (*Oryzias sinensis*) and Japanese ricefish (*Oryzias latipes*) were obtained from the Freshwater Bioresource Center at National Chiayi University. The AB strain zebrafish were obtained from the Taiwan Zebrafish Core Facility at Academia Sinica (http://icob.sinica.edu.tw/tzcas/). All fish in each experiment were mixed-gender of 6-10 months old adults in healthy condition. One month prior to the experiment, all tested fish species were reared in the centralized fish facility with a recirculating aquatic system at 28±1°C and a 10/14-h dark/light cycle. This process was important to eliminate some external factors that might affect their behavior performance, such as the stressful condition during the transfer [87]. The conductivity of the circulating system's water was kept between 300~1500 μS with pH 7.0-7.5. Ultraviolet (UV) light was utilized to filter the water constantly. All fish were held and raised in a trapezoid plastic tank with 34 cm at the top, 23 cm along the bottom, 19 cm along the diagonal side, 18 cm high, and 27 cm wide filled with 8 L of filtered water. Feed was given twice a day (09:00 and 17:00) with either lab-grown brine shrimp or commercial dry food. The general maintenance procedures and housing conditions were as previously described by Avdesh et al. [88].

4.2. Animal Ethics and Behavioral Tests

All fish experiments were performed following the guidelines issued by the Institutional Animal Care and Use Committees (IACUCs) of Chung Yuan Christian University (application number: CYCU106025, issue date 6 May 2018). All behavioral tests were conducted within the morning until afternoon (10:00 to 16:00), except for circadian locomotor activity rhythm, in a temperature-controlled room (26±1°C). After the acclimation process, a battery of behavioral tests, which were the novel tank, mirror biting, predator avoidance, conspecific social interaction, and shoaling tests based on the previous method, was conducted in all the fish groups [25]. The entire tests were done using a trapezoid tank with 22 cm along the bottom, 28 cm at the top, 15.2 cm high, and 15.9 cm along the diagonal side, and later, this tank was filled with ~1.25 L of circulating filtered water. A novel tank test was conducted to observe fish behavior as a response to a novel environment. Typically, fish spend most of the time at the bottom of the tank when introduced into a new environment and expand their swimming area to higher portions of the test tank after acclimating over time [89,90]. Next, a mirror biting test was done to evaluate the level of aggressiveness of fish. Generally, to drive away from the potential intruder, fish immediately display mirror biting behavior when introduced into a tank with a mirror. Afterward, to evaluate the fish's innate response to avoid its predator, a predator avoidance test was carried out. The Cichlid fish, *Amatitlania nigrofasciata*, was used as a presence of a predator. Later, social-related tests comprised of social interaction and shoaling tests were carried out between multiple fishes to observe their social behaviors. Generally, fish displays sociality with its conspecific in the social interaction test, while in the shoaling test, the fish capability to form a shoal was assessed. Next, the behavior tests were continued with the circadian locomotor activity rhythm test, which monitored its locomotor activity for 24 hours [91]. This test was conducted in a 30 × 30 × 7.5 cm acrylic tank filled with ~3 L of filtered water. A lightbox, which consisted of two types of a light source (Chip on board (COB) light-emitting diode (LED) and 940 nm infrared LED), was placed below the tank. All fish behavior videos were processed by computer with specification: Intel i7-5820K core @ 3.3GHz and 64 GB of RAM. Next, idTracker (http://www.idtracker.es/), open-source software, was used to collect and convert the fish movement data to trajectories [92]. All fish behavior tests were done in triplicate.

4.3. Brain Tissue Preparation, Total Protein Determination, and Quantification of Neurotransmitters, Stress Hormones, and Oxidative Stress Markers

In obtaining the brain tissues, immediate anesthesia and euthanasia were performed in medaka fishes by immersing them in tricaine solution (A5040, Sigma, St. Louis, MO, USA). Later, biochemical analyses were conducted on their whole brain tissue extract. Ice-cold phosphate-buffered saline (PBS) in volumes of 10 (v/w) was used to standardize a single homogenate of two to three whole medaka fish brains at pH 7.2. Afterward, to homogenize the tissue, a bullet blender (Next Advance, Inc., Troy, NY, USA) was utilized. After 15 minutes of 13000 rpm centrifugation, the supernatant was transferred to a sterilized microtube and stored at -20°C. Subsequently, Pierce BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Massachusetts, MA, USA) was applied to measure the total brain tissue's protein level. After the color was formed, it was analyzed by using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA) at 562 nm. Subsequently, all medaka fish brain tissues were analyzed to compare the differences of neurotransmitters, a stress hormone, and oxidative stress marker levels between each species of the medaka fishes. Several neurotransmitters, including serotonin (5-HT, ZGB-E1572), acetylcholine esterase (AChE, ZGB-E1637), dopamine (DA, ZGB-E1573), and norepinephrine (ZGB-E1571), were measured by target-specific ELISA kits. Meanwhile, tissue oxidative and anti-oxidative stress markers, reactive oxygen species (ROS) and catalase (CAT), respectively, and cortisol, one of the stress hormones, were also quantified by ELISA kits (ZGB-E1561, ZGB-E1598, and ZGB-E1575 Zgenebio Inc., Taipei, Taiwan). Later, a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA) was utilized to measure the absorbance at 450 nm and compared it to the standard curve to quantify the relative concentration of the target protein. Ten biological and three technical replicates were applied in the analysis (n = 30, except *O. latipes* (n = 29)).

4.4. Statistical Analyses

In determining the statistical difference between each group, statistical analyses were carried out using GraphPad Prism (GraphPad Software version 8 Inc., La Jolla, CA, USA). For all behavioral and biochemical data analyses except the novel tank test, Kruskal-Wallis with uncorrected Dunn's test was conducted to find the statistical differences in each group with every other group since the data are not normally distributed [93]. Meanwhile, two-way ANOVA with Geisser-greenhouse correction continued with uncorrected Fisher's LSD test was used for the novel tank test. Data for each fish group are expressed as either median with interquartile range or as the mean with a 95% confidence interval (CI) or standard deviation (SD). The statistic details for each behavioral and biochemical test are summarized in Table A2 and Table A3, respectively.

4.5. PCA, Heatmap, and Clustering Analysis

All the behavioral endpoint values from all the tested fish in every behavior test were input into a comma-separated values type file (.csv) using Microsoft Excel. All of the essential behavioral endpoints based on the prior study were listed and explained in Table A4 [25]. Next, the .csv file was uploaded to ClustVis (https://biit.cs.ut.ee/clustvis), a web tool designed to visualize and cluster multivariate data. Later, unit variance scaling for each row was carried out in order to treat each variable equally. Furthermore, singular value decomposition (SVD) with the imputation method was used to calculate principal components since there were no missing values in the dataset [94]. After data processing, PCA and heatmap results were exported and saved in the computer system.

4.6. Phylogenetic tree construction

The complete mitochondrial NADH dehydrogenase subunit 2 gene (nd2) sequences for each species of medaka fish were downloaded from the NCBI database with the accession numbers NC_012976 (*O. dancena*), NC_004387 (*O. latipes*), LC051726 (*O. woworae*), and NC_013434 (*O. sinensis*). The gene sequences of nd2 of *D. rerio* (NC_002333), representing outgroup species, were also downloaded from NCBI database. All nucleotide sequences nd2 of all species described above were then aligned using Geneious software (Biomatters, Auckland, New Zealand). Subsequently, phylogenetic analysis was also conducted with Geneious software with default parameter settings.

5. Conclusions

To sum up, the current study demonstrated that phenotypes of four medaka fish, which were *O. latipes*, *O. dancena*, *O. woworae*, and *O. sinensis*, were different in their behavior in various behavioral tests. These differences might be related to the different basal levels of several neurotransmitters, the stress hormone, and oxidative stress markers measured. Furthermore, while our phenomics-based hierarchical cluster results showed a similar interspecies relationship between zebrafish and medaka fishes to the phylogenetic tree, the interspecies comparison between each medaka fish of these two approaches demonstrated a slight difference relationship result between three medaka fishes, suggesting that a re-interpretation of some findings in comparative biology might be required. Our findings also suggest that many challenges remain to be addressed before robustly use behavioral endpoints for ecological hazard evaluation since the instability of some behavior performances can be observed in the medaka fishes. We believe that our results contribute to the growing database of phenotypical differences between several medaka fish species and provide one of the foundations for future phenomics studies of medaka fish. Furthermore, considering the abundance of medaka fish strain listed in National BioResource Project (NBRP) Medaka, it is intriguing to study the differences between each medaka strain in every aspect, including behavior and biochemical [95].

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Institutional Review Board Statement: The study was conducted according to the guidelines approved by the Institutional Animal Care and Use Committees (IACUCs) of Chung Yuan Christian University (application number: CYCU106025, issue date 6 May 2018).

Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

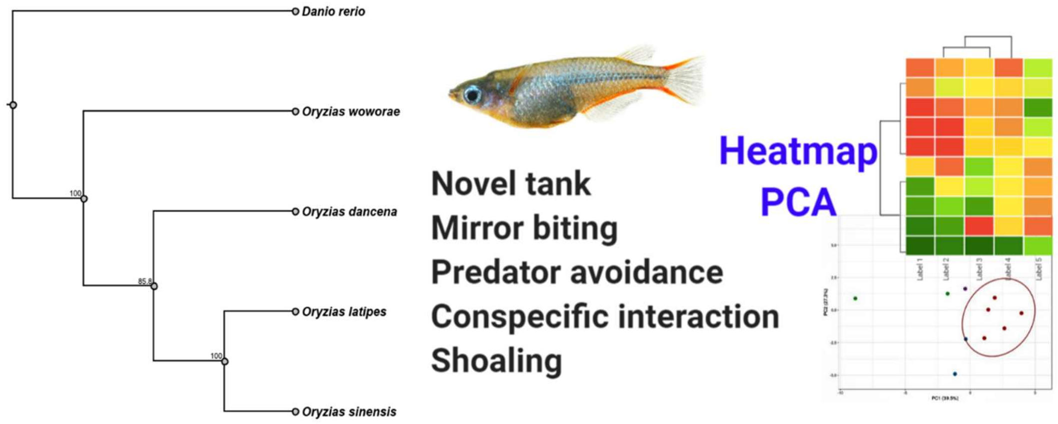

Figure A1. Overview of the experimental design in the present study.

Table A1. The individual P-value of each fish behavioral endpoint comparison in the novel tank test. The P-values were obtained from a two-way ANOVA test with Geisser-greenhouse correction continued with uncorrected Fisher's LSD test.

Behavioral Endpoints		*O. dancena*	*O. latipes*	*O. woworae*	*O. sinensis*	*D. rerio*
	O. dancena		0.0097	0.0005	< 0.0001	< 0.0001

Average Speed	*O. latipes*	0.0097		< 0.0001	0.1134	< 0.0001
	O. woworae	0.0005	< 0.0001		< 0.0001	< 0.0001
	O. sinensis	< 0.0001	0.1134	< 0.0001		< 0.0001
	D. rerio	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
Freezing Time Movement Ratio	*O. dancena*		0.2654	< 0.0001	0.0155	< 0.0001
	O. latipes	0.2654		0.0002	0.0002	< 0.0001
	O. woworae	< 0.0001	0.0002		< 0.0001	< 0.0001
	O. sinensis	0.0155	0.0002	< 0.0001		< 0.0001
	D. rerio	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
Swimming Time Movement Ratio	*O. dancena*		0.1090	0.0002	0.0286	< 0.0001
	O. latipes	0.1090		0.0171	< 0.0001	< 0.0001
	O. woworae	0.0002	0.0171		< 0.0001	< 0.0001
	O. sinensis	0.0286	< 0.0001	< 0.0001		< 0.0001
	D. rerio	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
Rapid Time Movement Ratio	*O. dancena*		< 0.0001	< 0.0001	0.0007	< 0.0001
	O. latipes	< 0.0001		< 0.0001	0.3643	< 0.0001
	O. woworae	< 0.0001	< 0.0001		< 0.0001	0.4803
	O. sinensis	0.0007	0.3643	< 0.0001		< 0.0001
	D. rerio	< 0.0001	< 0.0001	0.4803	< 0.0001	
Time in Top	*O. dancena*		0.0636	0.0595	< 0.0001	0.0164
	O. latipes	0.0636		0.9472	< 0.0001	< 0.0001
	O. woworae	0.0595	0.9472		< 0.0001	< 0.0001
	O. sinensis	< 0.0001	< 0.0001	< 0.0001		< 0.0001
	D. rerio	0.0164	< 0.0001	< 0.0001	< 0.0001	
Number of Entries to The Top	*O. dancena*		0.0050	< 0.0001	0.4143	0.0619
	O. latipes	0.0050		< 0.0001	0.0461	< 0.0001
	O. woworae	< 0.0001	< 0.0001		< 0.0001	< 0.0001
	O. sinensis	0.4143	0.0461	< 0.0001		0.0057
	D. rerio	0.0619	< 0.0001	< 0.0001	0.0057	
Latency to Enter the Top	*O. dancena*		0.2674	0.4705	< 0.0001	< 0.0001
	O. latipes	0.2674		0.0648	< 0.0001	< 0.0001
	O. woworae	0.4705	0.0648		< 0.0001	0.0002
	O. sinensis	< 0.0001	< 0.0001	< 0.0001		0.5059
	D. rerio	< 0.0001	< 0.0001	0.0002	0.5059	
Total Distance Traveled in The Top	*O. dancena*		0.1233	0.2273	0.0753	< 0.0001
	O. latipes	0.1233		0.0061	0.0009	< 0.0001
	O. woworae	0.2273	0.0061		0.5744	< 0.0001
	O. sinensis	0.0753	0.0009	0.5744		< 0.0001
	D. rerio	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
Thigmotaxis	*O. dancena*		0.0162	< 0.0001	< 0.0001	< 0.0001
	O. latipes	0.0162		< 0.0001	< 0.0001	0.0005
	O. woworae	< 0.0001	< 0.0001		0.2250	< 0.0001

	O. sinensis	< 0.0001	< 0.0001	0.2250		< 0.0001
	D. rerio	< 0.0001	0.0005	< 0.0001	< 0.0001	

Table A2. The individual P-value of each fish behavioral endpoint comparison in behavior tests. The P-values were obtained from the Kruskal-Wallis test continued with uncorrected Dunn's LSD test.

Behavior Tests	Behavioral Endpoints		*O. dancena*	*O. latipes*	*O. woworae*	*O. sinensis*	*D. rerio*
Mirror Biting Test	Mirror Biting Time Percentage	*O. dancena*		0.0988	0.3011	0.0040	0.1159
		O. latipes	0.0988		0.0073	<0.0001	0.9375
		O. woworae	0.3011	0.0073		0.0542	0.0091
		O. sinensis	0.0040	<0.0001	0.0542		<0.0001
		D. rerio	0.1159	0.9375	0.0091	<0.0001	
	Longest Duration in the Mirror Side Percentage	*O. dancena*		0.7656	0.0466	0.0023	0.4318
		O. latipes	0.7656		0.0907	0.0054	0.2782
		O. woworae	0.0466	0.0907		0.2210	0.0055
		O. sinensis	0.0023	0.0054	0.2210		0.0002
		D. rerio	0.4318	0.2782	0.0055	0.0002	
Predator Avoidance Test	Approaching Predator Time Percentage	*O. dancena*		0.7111	0.1669	0.0016	0.0104
		O. latipes	0.7111		0.3115	0.0044	0.0285
		O. woworae	0.1669	0.3115		0.0448	0.2384
		O. sinensis	0.0016	0.0044	0.0448		0.3064
		D. rerio	0.0104	0.0285	0.2384	0.3064	
	Average Distance to the Predator's Separator	*O. dancena*		0.1360	0.0153	0.0017	0.1091
		O. latipes	0.1360		<0.0001	<0.0001	0.0020
		O. woworae	0.0153	<0.0001		0.2628	0.4109
		O. sinensis	0.0017	<0.0001	0.2628		0.0710
		D. rerio	0.1091	0.0020	0.4109	0.0710	
Social Interaction Test	Conspecific Interaction Time Percentage	*O. dancena*		0.0204	0.0675	0.5032	0.0155
		O. latipes	0.0204		<0.0001	0.1432	<0.0001
		O. woworae	0.0675	<0.0001		0.0187	0.5531
		O. sinensis	0.5032	0.1432	0.0187		0.0038
		D. rerio	0.0155	<0.0001	0.5531	0.0038	
	Average Distance to the Conspecific's Separator	*O. dancena*		0.2037	0.0349	0.4902	0.0294
		O. latipes	0.2037		0.0007	0.6318	0.0006
		O. woworae	0.0349	0.0007		0.0085	0.9450
		O. sinensis	0.4902	0.6318	0.0085		0.0071
		D. rerio	0.0294	0.0006	0.9450	0.0071	
	Longest Conspecific Interaction Percentage	*O. dancena*		0.0969	0.0360	0.7053	0.2989
		O. latipes	0.0969		0.0002	0.2506	0.0070
		O. woworae	0.0360	0.0002		0.0211	0.2902
		O. sinensis	0.7053	0.2506	0.0211		0.1823
		D. rerio	0.2989	0.0070	0.2902	0.1823	

Shoaling Test	Average Inter-fish Distance	*O. dancena*		0.3414	<0.0001	0.0014	<0.0001
		O. latipes	0.3414		0.0021	0.0193	0.0023
		O. woworae	<0.0001	0.0021		0.6498	0.9773
		O. sinensis	0.0014	0.0193	0.6498		0.6685
		D. rerio	<0.0001	0.0023	0.9773	0.6685	
	Average Shoal Area	*O. dancena*		0.1811	0.0043	0.0036	0.0010
		O. latipes	0.1811		0.1294	0.0888	0.0503
		O. woworae	0.0043	0.1294		0.7447	0.6597
		O. sinensis	0.0036	0.0888	0.7447		0.9410
		D. rerio	0.0010	0.0503	0.6597	0.9410	
	Average Nearest Neighbor Distance	*O. dancena*		0.0654	<0.0001	0.0001	<0.0001
		O. latipes	0.0654		0.0056	0.0297	0.0066
		O. woworae	<0.0001	0.0056		0.7350	0.9572
		O. sinensis	0.0001	0.0297	0.7350		0.7721
		D. rerio	<0.0001	0.0066	0.9572	0.7721	
	Average Farthest Neighbor Distance	*O. dancena*		0.5503	<0.0001	0.0001	<0.0001
		O. latipes	0.5503		0.0006	0.0009	0.0008
		O. woworae	<0.0001	0.0006		0.8418	0.9270
		O. sinensis	0.0001	0.0009	0.8418		0.7774
		D. rerio	<0.0001	0.0008	0.9270	0.7774	
Circadian Locomotor Activity Rhythm – Day Cycle	Average Speed	*O. dancena*		0.0432	0.0403	<0.0001	<0.0001
		O. latipes	0.0432		<0.0001	<0.0001	<0.0001
		O. woworae	0.0403	<0.0001		<0.0001	<0.0001
		O. sinensis	<0.0001	<0.0001	<0.0001		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
	Average Angular Velocity	*O. dancena*		0.4015	0.9343	<0.0001	<0.0001
		O. latipes	0.4015		0.3569	<0.0001	<0.0001
		O. woworae	0.9343	0.3569		<0.0001	<0.0001
		O. sinensis	<0.0001	<0.0001	<0.0001		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
	Meandering	*O. dancena*		0.0603	0.0097	0.5545	<0.0001
		O. latipes	0.0603		<0.0001	0.2761	<0.0001
		O. woworae	0.0097	<0.0001		0.0037	<0.0001
		O. sinensis	0.5545	0.2761	0.0037		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
	Freezing Movement Time Ratio	*O. dancena*		0.3357	0.3036	<0.0001	<0.0001
		O. latipes	0.3357		0.0464	<0.0001	<0.0001
		O. woworae	0.3036	0.0464		<0.0001	<0.0001
		O. sinensis	<0.0001	<0.0001	<0.0001		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
		O. dancena		0.1337	0.2348	<0.0001	<0.0001
		O. latipes	0.1337		0.0072	<0.0001	0.0086

	Swimming Movement Time Ratio	*O. woworae*	0.2348	0.0072		<0.0001	<0.0001
		O. sinensis	<0.0001	<0.0001	<0.0001		<0.0001
		D. rerio	<0.0001	0.0086	<0.0001	<0.0001	
	Rapid Movement Time Ratio	*O. dancena*		0.6290	<0.0001	0.0087	<0.0001
		O. latipes	0.6290		<0.0001	0.0022	<0.0001
		O. woworae	<0.0001	<0.0001		0.0066	<0.0001
		O. sinensis	0.0087	0.0022	0.0066		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
Circadian Locomotor Activity Rhythm – Night Cycle	Average Speed	*O. dancena*		0.0602	0.0013	<0.0001	<0.0001
		O. latipes	0.0602		<0.0001	0.0043	<0.0001
		O. woworae	0.0013	<0.0001		<0.0001	<0.0001
		O. sinensis	<0.0001	0.0043	<0.0001		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
	Average Angular Velocity	*O. dancena*		0.1734	0.0331	0.2380	<0.0001
		O. latipes	0.1734		0.0005	0.9701	<0.0001
		O. woworae	0.0331	0.0005		0.0020	<0.0001
		O. sinensis	0.2380	0.970	0.0020		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
	Meandering	*O. dancena*		0.4948	0.0310	<0.0001	<0.0001
		O. latipes	0.4948		0.0045	0.0002	<0.0001
		O. woworae	0.0310	0.0045		<0.0001	<0.0001
		O. sinensis	<0.0001	0.0002	<0.0001		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
	Freezing Movement Time Ratio	*O. dancena*		0.0119	0.0208	<0.0001	<0.0001
		O. latipes	0.0119		<0.0001	0.0012	<0.0001
		O. woworae	0.0208	<0.0001		<0.0001	<0.0001
		O. sinensis	<0.0001	0.0012	<0.0001		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
	Swimming Movement Time Ratio	*O. dancena*		0.0106	0.0226	<0.0001	<0.0001
		O. latipes	0.0106		<0.0001	0.0009	<0.0001
		O. woworae	0.0226	<0.0001		<0.0001	<0.0001
		O. sinensis	<0.0001	0.0009	<0.0001		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	
	Rapid Movement Time Ratio	*O. dancena*		0.0834	<0.0001	0.0279	<0.0001
		O. latipes	0.0834		<0.0001	0.5159	<0.0001
		O. woworae	<0.0001	<0.0001		0.0005	<0.0001
		O. sinensis	0.0279	0.5159	0.0005		<0.0001
		D. rerio	<0.0001	<0.0001	<0.0001	<0.0001	

Table A3. The individual P-value of each medaka fish's neurotransmitters, antioxidant activity, and oxidative stress content comparisons in the biochemical tests. The P-values were obtained from a Kruskal-Wallis test continued with uncorrected Dunn's test.

Biomarkers	Medaka Fishes	*O. dancena*	*O. latipes*	*O. woworae*	*O. sinensis*

5-HT	*O. dancena*		0.0407	0.1569	0.0488
	O. latipes	0.0407		0.0005	0.939
	O. woworae	0.1569	0.0005		0.0007
	O. sinensis	0.0488	0.939	0.0007	
CAT	*O. dancena*		0.003	0.8633	0.0093
	O. latipes	0.0030		0.0052	0.7163
	O. woworae	0.8633	0.0052		0.0151
	O. sinensis	0.0093	0.7163	0.0151	
NE	*O. dancena*		0.0036	0.5032	0.0583
	O. latipes	0.0036		0.0003	0.3107
	O. woworae	0.5032	0.0003		0.0104
	O. sinensis	0.0583	0.3107	0.0104	
DA	*O. dancena*		0.0059	0.3199	0.0322
	O. latipes	0.0059		0.0002	0.5405
	O. woworae	0.3199	0.0002		0.0017
	O. sinensis	0.0322	0.5405	0.0017	
Cortisol	*O. dancena*		0.0116	0.2138	0.0168
	O. latipes	0.0116		0.0002	0.8935
	O. woworae	0.2138	0.0002		0.0003
	O. sinensis	0.0168	0.8935	0.0003	
AChE	*O. dancena*		0.0389	0.1308	0.3293
	O. latipes	0.0389		0.0003	0.2756
	O. woworae	0.1308	0.0003		0.0129
	O. sinensis	0.3293	0.2756	0.0129	
ROS	*O. dancena*		0.0217	0.0692	0.0692
	O. latipes	0.0217		<0.0001	0.6325
	O. woworae	0.0692	<0.0001		0.0003
	O. sinensis	0.0692	0.6325	0.0003	

Table A4. Summary of fish behavioral endpoints measured in each behavioral test during this experiment.

Index	Behavior endpoints (units)	Definition	Applied to
1-1-1 & 1-2-1	Average speed (cm s^{-1})	Total distance traveled by fish divided by total time duration	**Novel Tank Test**
1-1-2 & 1-2-2	Freezing time movement ratio (%)	Total percentage of time when fish's speed less than 1 cm s^{-1}	
1-1-3 & 1-2-3	Swimming time movement ratio (%)	Total percentage of time when fish's speed is between 1 and 10 cm s^{-1}	
1-1-4 & 1-2-4	Rapid movement ratio (%)	Total percentage of time when fish's speed more than 10 cm s^{-1}	
1-1-5 & 1-2-5	Time in top duration (%)	Total time spent in the top portion of the novel tank in percentage	

1-1-6 & 1-2-6	Number of entries to the top	Total times fish enter the upper half of the tank	
1-1-7 & 1-2-7	Latency to enter the top (s)	The amount of time it takes the fish to cross into the upper half of the tank	
1-1-8 & 1-2-8	Total distance traveled in the top (cm)	Total distance traveled in the top portion of the novel tank	
1-1-9 & 1-2-9	Thigmotaxis (cm)	The average distance of the fish from the center of the tank	
2-1	Mirror biting time percentage (%)	Total percentage of time when fish stayed in the mirror biting zone	**Mirror Biting Test**
2-2	Longest duration in the mirror side percentage (%)	Total percentage of fish longest duration stayed in the mirror biting zone	
3-1	Approaching predator time percentage (%)	Total percentage of time when fish stayed in the approaching predator zone	**Predator Avoidance Test**
3-2	Average distance to the predator's separator (cm)	Average distance of fish to the predator's separator	
4-1	Conspecific interaction time percentage (%)	Total percentage of time when fish stayed in the conspecific interaction zone	**Social Interaction Test**
4-2	Average distance to the conspecific's separator (cm)	Average distance of fish to the conspecific's separator	
4-3	Longest conspecific interaction percentage (%)	Total percentage of fish longest duration stayed in the conspecific interaction	
5-1	Average inter-fish distance (cm)	Average distance between the body center of every member of the shoal	**Shoaling Test**
5-2	Average shoal area (cm^2)	Average size of the shoal	
5-3	Average nearest neighbor distance (cm)	Distance for the body center of each fish to the closest neighboring fish	
5-4	Average farthest neighbor distance (cm)	Distance for the body center of each fish to the farthest neighboring fish	
6-1-1 & 6-2-1	Average speed (cm s^{-1})	Total distance traveled by fish divided by total time duration	**Circadian Locomotor Activity Rhythm**
6-1-2 & 6-2-2	Average Angular Velocity (°/s)	Total turning angle of fish divided by total time duration	
6-1-3 & 6-2-3	Meandering (°/m)	Total turning angle of fish divided by total distance traveled by fish	
6-1-4 & 6-2-4	Freezing time movement ratio (%)	Total percentage of time when fish's speed less than 1 cm s^{-1}	
6-1-5 & 6-2-5	Swimming time movement ratio (%)	Total percentage of time when fish's speed is between 1 and 10 cm s^{-1}	
6-1-6 & 6-2-6	Rapid movement ratio (%)	Total percentage of time when fish's speed more than 10 cm s^{-1}	

Table A5. Summary table of each strain coefficient of variation in each behavior endpoint.

Index	**Coefficient of Variation (%)**				
	Danio rerio	*Oryzias dancena*	*Oryzias latipes*	*Oryzias woworae*	*Oryzias sinensis*
1-1-1	30.18	76.43	81.78	70.83	64.87
1-1-2	85.28	67.35	50.16	66.54	50.09
1-1-3	11.72	50.75	39.62	40.12	51.07
1-1-4	119.26	183.56	300.39	127.78	151.28
1-1-5	94.04	125.63	163.79	130.83	69.09
1-1-6	82.26	122.4	156.09	125.42	90.9
1-1-7	150.18	102.78	74.5	90.45	180.32
1-1-8	99.42	127.26	173.59	139.06	81.21
1-1-9	33.2	33.02	33.81	31.92	37.59
1-2-1	36.2	64.82	57.81	43.05	61.88
1-2-2	71.59	80.08	70.07	72.67	57.6
1-2-3	8.83	47.66	32.26	19.36	46.96
1-2-4	153.56	149.89	223.11	107.58	173.84
1-2-5	61.45	92.21	83.51	69.71	62.6
1-2-6	78.05	91.71	89.13	67.03	88.95
1-2-7	164.92	136.08	142.32	156.62	207.24
1-2-8	70.03	96.2	106.1	74.88	70.96
1-2-9	35.58	36.74	30.87	31.25	28.83
2-1	47.42	60.31	64.69	36.62	42.34
2-2	83.05	104.5	88.44	71.91	79.35
3-1	137	96.47	128.2	160.5	226
3-2	13.84	32.45	28.23	25.17	21.64
4-1	33.86	45.1	81.95	43.7	68.01
4-2	55.24	45.46	31.03	72.45	55.06
4-3	96.13	109.6	131.6	94.31	92.97
5-1	20.73	47.48	31.46	27.87	39.97
5-2	46.02	66.53	56.85	54.23	76.82
5-3	20.73	51.52	28.68	27.49	44.87
5-4	19.33	34.87	27	25.25	28.02
6-1-1	43.78	47.25	64.57	45	61.46
6-1-2	44.33	45.96	51.86	50.71	71.5
6-1-3	71.4	46.4	51.83	48.35	58.84
6-1-4	205.7	47.12	52.79	52.03	36.53
6-1-5	42.51	37.84	48.91	38.6	73.53
6-1-6	49.05	265	293.3	177.9	171.9
6-2-1	70.4	57.58	69.35	76.38	42.71
6-2-2	62.52	47.03	49.12	44.53	50.04
6-2-3	64.63	51.86	51.74	64.83	54.78
6-2-4	68.4	32.2	28.96	47.31	11.73
6-2-5	38.88	77.81	91.62	73.25	72.81

6-2-6	170.9	217.7	225.2	217.1	220.2
Average	**70.52683**	**81.77098**	**89.90951**	**74.16073**	**79.91122**

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