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The Medaka approach to evolutionary social neuroscience

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ABSTRACT

Previously, the integration of comparative biological and neuroscientific approaches has led to significant advancements in social neuroscience. This review highlights the potential and future directions of evolutionary social neuroscience research utilizing medaka fishes (the family Adrianichthyidae) including Japanese medaka (*Oryzias latipes*). We focus on medaka social cognitive capabilities and mate choice behavior, particularly emphasizing mate preference using visual cues. Medaka fishes are also advantageous due to their abundant genetic resources, extensive genomic information, and the relative ease of laboratory breeding and genetic manipulation. Here we present some research examples of both the conventional neuroscience approach and evolutionary approach involving medaka fishes and other species. We also discuss the prospects of uncovering the molecular and cellular mechanisms underlying the diversity of visual mate preference among species. Especially, we introduce that the single-cell transcriptome technology, particularly in conjunction with 'Adaptive Circuitry Census', is an innovative tool that bridges comparative biological methods and neuroscientific approaches. Evolutionary social neuroscience research using medaka has the potential to unveil fundamental principles in neuroscience and elucidate the mechanisms responsible for generating diversity in mating strategies.

1. Introduction

Both neuroscientists and evolutionary ecologists have extensively been interested in diversified and complex animal behaviors since the era of Darwin (Darwin, 1871, Darwin, 1897). However, their research methodologies significantly differ (Jourjine and Hoekstra, 2021). Neuroscience has traditionally concentrated on a limited number of genetically tractable model organisms (e.g., mice, zebrafish, fruit flies, and nematodes) to facilitate reproducible experiments under controlled laboratory conditions. This focus has led to the development of standardized toolkits and a profound understanding of mechanisms ranging from gene expression to neural circuits and behavior (Fig. 1A). Conversely, evolutionary ecology has aimed to elucidate the processes of behavioral diversification by comparing multiple species or lineages with shared ancestors. Nonetheless, there has been a lack of appropriate research strategies to elucidate the molecular and neural mechanisms driving behavioral differences among extant species

(lineages/populations) in natural environments. Integrating these two disparate approaches to simultaneously elucidate the proximate (mechanistic) and ultimate (evolutionary) causes of diverse and complex animal behaviors has been a long-standing objective for many neuroethologists as proposed in "Tinbergen's four questions" (Tinbergen, 1963; Bateson and Laland, 2013).

The integration of comparative biological and neuroscientific approaches has also led to significant advancements in the field of social neuroscience (Comparative social neuroscience). Several studies have demonstrated how this interdisciplinary approach has revolutionized our understanding of social behavior at the neural level. For instance, social neuropeptides such as oxytocin and vasopressin were initially characterized as hormones operating in peripheral organs. However, recent research has elucidated their pivotal roles in social cognition and behavioral selection across diverse phylogenetic taxa, ranging from fish to mammals (Walum and Young, 2018; Yokoi et al., 2020; Akinrinade et al., 2023). The paradigm shift in this field was precipitated by a

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Review Article







Fig. 1. A concept of evolutionary social neuroscience research using medaka fishes. (A) Conventional Neuroscience Approach. The Cab and d-rR strains of Japanese medaka (*Oryzias latipes*) are representative laboratory strains. Genetic tools are employed to manipulate laboratory strains at both genetic and neuronal levels. These genetically manipulated strains are then compared with controls to elucidate the functions of specific genes and neurons. (B) Evolutionary Social Neuroscience Approach. By comparing genetically distinct groups (e.g. species, populations, and strains) at behavioral, genetic, and neuronal levels, this approach aims to identify genetic changes that are responsible for behavioral diversity in natural environments. This will enable the understanding of the evolutionary processes of how and when the diversity emerged. Genetic tools are utilized to recreate natural variation, thereby elucidating the genetic and neural foundations that give rise to such behavioral diversity. The image of medaka fish is from TogoTV © 2016 DBCLS TogoTV, CC-BY-4.0.

seminal comparative biological study. This investigation juxtaposed the monogamous prairie vole (Microtus ochrogaster) with its promiscuous congener, the meadow vole (M. pennsylvanicus). Dr. Larry Young and his colleagues elucidated that the brain functions of social neuropeptides play a central role in establishing monogamy (pair-bonding) in prairie voles. Through interspecific comparison with the promiscuous meadow vole, they demonstrated that species-specific and individual variations in mating strategies correlate with distinct expression patterns of social neuropeptide receptors. Moreover, they empirically demonstrated that manipulating the expression of a single receptor gene (avpr1a) in a specific brain region could induce a phenotypic shift in the social behavior of promiscuous meadow voles, recapitulating a prairie vole-like (monogamous) behavioral pattern (Lim et al., 2004). While mating and social behaviors often involve complex polygenic interactions, this exemplifies that monogenic effects can engender species-specific differences (Fig. 1B). The field of behavioral genetics has seen significant advancements (Jourjine and Hoekstra, 2021). However, in this previous study, it was difficult to investigate the phylogenetic history of the natural variation that arose for adaptation. Specifically, it was challenging to determine when and how these differences emerged during the evolutionary process. This review will explain how to bridge this gap by leveraging medaka, an established model organism in molecular genetics, to synthesize comparative biological and neuroscientific approaches.

Recently, next-generation sequencing has enabled the identification of genes responsible for lineage/population-specific phenotypic differences using population genetic approaches. To identify the genetic basis of intraspecific lineage/population differences, it is important to select lineages or populations with high phenotypic diversity but low genetic variation (within each population) as experimental subjects. This is to minimize false positives caused by genetic polymorphisms unrelated to the traits of interest. Therefore, lineages/populations that have diverged

from a common ancestor while maintaining high phenotypic diversity are ideal comparative subjects. Specifically, species that experienced genetic bottlenecks during migration from the continent, and subsequently expanded across diverse ecological niches within peninsulas or archipelagos from the continent, make excellent subjects for research. A notable example in evolutionary genetics research involves the local populations of Arabidopsis thaliana that underwent genetic bottlenecks during their migration from the Eurasian continent to the Scandinavian peninsula, followed by a latitudinal expansion of their habitat (Long et al., 2013). This study successfully identified genes associated with flowering time phenology, which were not detectable in continental A. thaliana local populations (Sasaki et al., 2021). The enhanced detection capability in the Scandinavian populations can be attributed to the genetic bottleneck effect and subsequent adaptation to diverse ecological niches. Similarly, some flora of the Japanese archipelago, having likely experienced genetic bottlenecks during continental-insular migration, present an excellent model system for population genetics studies. For instance, the leguminous plant Lotus japonicus (Miyako-gusa) colonized Kyushu approximately 20 M years ago and subsequently expanded its distribution throughout Japan (Shah et al., 2020). In Miyako-gusa, it was revealed that genes associated with winter hardiness and flowering regulation were key to the local adaptation of naturally occurring strains in Japan.

Japanese medaka shares a similar evolutionary history with Miyakogusa. The species comprises two distinct populations: Southern and Northern Japanese medaka. Southern Japanese medaka originated in northern Kyushu before expanding across Japan, while the Tajima-Tango region is the ancestral origin of Northern Japanese medaka (Fig. 2, Katsumura et al., 2019). Moreover, related medaka species endemic to Sulawesi, Indonesia, have undergone rapid radiation in the island's ancient lakes from a common ancestor (Mokodongan and Yamahira, 2015). This review will introduce how to advance research on the genomic foundations of phenotypic variations in social behaviors by studying the medaka genus with this unique phylogeographic history.

1.1. Advantages of using medaka

- Rich Genetic Resources: National BioResource Project (NBRP) medaka in Japan (https://shigen.nig.ac.jp/medaka/) maintains and distributes over 100 local populations of Japanese medaka (Fig. 2). A near-isogenic panel of 80 inbred lines, the Medaka Inbred Kiyosu-Karlsruhe (MIKK) panel, is also available (Fitzgerald et al., 2022). Among ~40 related species (the family Adrianichthyidae), a variety (>20) of species endemic to Sulawesi, Indonesia (Fig. 3), showing a significant phenotypic diversity, is available.
- 2. Comprehensive Genomic Information: The reference genomes of Japanese medaka (O. latipes species complex; Ichikawa et al., 2017) and closely related species have been already sequenced and assembled (Kim et al., 2018, Takehana et al., 2020, Ansai et al., 2021). We have sequenced all of the wild-derived strains of Japanese medaka and are now preparing the genome database (https://me dakabase.nbrp.jp/), revealing their diversification processes.
- 3. Ease of Laboratory Breeding and Genetic Manipulation: Both Japanese and Indonesian medaka can be readily bred in laboratories and are amenable to state-of-the-art gene editing technologies (Kinoshita et al., 2009, Ansai et al., 2021, Hara et al., 2023)

These attributes enable the fusion of neuroscientific and evolutionary approaches using medaka, facilitating the establishment of a new research field termed "medaka evolutionary social neuroscience," which aims to comprehensively analyze the diversification mechanisms of social cognitive abilities and reproductive behaviors in vertebrates (Fig.1B).



Fig. 2. Diversity of wild-derived strains in Japanese medaka and relatives (*Oryzias latipes* species complex). (A) A map showing the original collection sites of wild-derived strains in East Asia. The sites of four species of *O. latipes* species complex (*O. latipes*, *O. sakaizumii*, *O. sinensis*, and *Oryzias* sp.) and an outgroup sister species (*O. curvinotus*) were shown. (B) A maximum likelihood phylogenetic tree of 81 stocks of the wild-derived strains inferred by nuclear DNA sequences. S.JPN: Southern Japanese group (*O. latipes*), N.JPN: Northern Japanese group (*O. sakaizumii*), E.KOR: Eastern Korean group (*Oryzias* sp.), W.KOR: Western Korean and Chinese group (*O. sinensis*), Taj-Tan: Tajima-Tango group (*O. sakaizumii*). The southern Japanese group (*O. latipes*) originated in northern Kyushu region, while the northern Japanese group (*O. sakaizumii*), originated in the Tajima-Tango region. Modified from Fig. 3 in Katsumura et al., (2019) (G3: 9, 217–228), CC-BY-4.0.

2. Characteristics of Japanese medaka as a laboratory experimental model

Some laboratory strains (Fig. 1A) of Japanese medaka (*Oryzias latipes*) have served as a model fish species for molecular genetics, alongside zebrafish (Furutani-Seiki and Wittbrodt, 2004). Both species possess embryos, larvae, and adults of comparable sizes, making them suitable for experimental manipulation. From an evolutionary perspective, zebrafish and medaka were diverged from each other approximately 314–332 million years ago (Kasahara et al., 2007), resulting in different strategies in reproductive behaviors. Notably, female medaka have a 24-hour ovarian cycle, markedly shorter than the 5-day cycle of zebrafish (Hisaoka and Firlit, 1962). This characteristic allows for the daily analysis of reproductive behavior using the same individuals and the examination of social interactions between conspecifics and between sexes using females in synchronized physiological states, providing a significant advantage for social neuroscience research (Okuyama et al., 2014; Yokoi et al., 2015; Daimon et al., 2022). The medaka reproductive behavior consists of a series of stereotyped actions: approach, courtship display, wrapping, and spawning (Ono and Uematsu, 1957). The high



Fig. 3. Diversity of medaka fishes endemic to Sulawesi, Indonesia. (A) A map showing sampling sites of 17 endemic species. The map was provided by Thomas von Rintelen. (B) A phylogenetic tree based on whole-genome sequencing data. The tree was inferred using 10,174 single copy orthologs with a maximum likelihood method. Open arrowhead showed *Oryzias woworae*, while closed arrowhead *O. celebensis*. These two species are phylogenetically proximate, enabling interspecific hybridization for QTL analysis. (C) Representative images of adult males and females of *O. woworae*. The males possess red pectoral fins. (D) Representative images of adult males and females of *O. celebensis*. The males possess black patterns mainly on their tail fins, which appear/disappear rapidly in a context-dependent manner. (A-B) were from Fig. 1 and Fig. 2a in Ansai et al., (2021) (*Nat. Comm.* 12, 1350, CC-BY-4.0) while (D) was from Ueda et al., (2024) (*Biol. Lett.*, CC-BY-4.0).

reproducibility of this behavioral pattern makes it suitable for quantitative behavioral analysis. Furthermore, female medaka prefer specific mating partners based on visual familiarity (Okuyama et al., 2014; Yokoi et al., 2016; Wang and Takeuchi, 2017; Yokoi et al., 2020), which is advantageous for studying the neural mechanisms of visual information processing and social cognition. While zebrafish primarily engage in group spawning induced by pheromones (olfactory cues) (Hutter et al., 2010; Yabuki et al., 2016), medaka perform mate choice through visual cues. This characteristic affords medaka a unique advantage in research focused on social cognition mediated by visual information processing.

3. Social cognitive abilities and mate preference in Japanese Medaka

Japanese medaka (Oryzias latipes) exhibit advanced social cognitive abilities, particularly in familiarity recognition and mate choice through visual cues (Okuyama et al., 2014; Yokoi et al., 2016; Wang and Takeuchi, 2017; Yokoi et al., 2020). Visual familiarization, the process of becoming acquainted with individuals through visual contact, has been shown to significantly influence mate preference in female medaka. Preference for familiar males has been demonstrated primarily through two experimental paradigms (Yokoi et al., 2022). In the first experiment, male-female pairs are allowed visual contact through a transparent partition before mating testing. The visual familiarization significantly increases female receptivity towards the familiarized male, decreasing the latency to mate. The second experiment simulates a more natural competitive situation. In this triadic relationship paradigm (two males and one female), mate choice behavior can be directly observed (Okuyama et al., 2014; Yokoi et al., 2015, 2016). Interestingly, when one of the two males is visually familiarized with the female in advance, about 90 % of the offspring are derived from that familiarized male (Okuyama et al., 2014). These experimental results clearly demonstrate that female medaka can discriminate between familiar and unfamiliar males based on visual information and show a strong preference for them. This ability is thought to contribute to adaptive mate choice in natural environments (Yokoi et al., 2016).

Familiarity recognition ability is not limited to medaka but has been widely observed in many fish species (Griffiths and Ward, 2011; Ward et al., 2020). Preference for shoaling with familiar conspecifics has been confirmed in a wide range of fish species, including guppies (Magurran et al., 1994), sticklebacks (Barber and Ruxton, 2000), minnows (Chivers et al., 1995), shiners (Farmer et al., 2004), cichlids (Lee-Jenkins and Godin, 2013), zebrafish (Ribeiro et al., 2020), and even sharks (Keller et al., 2017). In some fish species, familiarity-based mate choice has also been observed. For example, guppies (Poecilia reticulata) have the ability to recognize familiar conspecifics based on past interaction experiences (Ward et al., 2020). Interestingly, male guppies display courtship more frequently towards unfamiliar females than familiar ones. This behavior may promote the dispersal of male genes and lead to offspring with more diverse genetic backgrounds (Hughes et al., 1999; Mariette et al., 2010). Furthermore, some fish species, including medaka and cichlids, have been reported to have the ability to identify individuals based on visual stimuli from "the facial region" (Kohda et al., 2015; Wang and Takeuchi, 2017). For instance, Astatotilapia burtoni, a cichlid species inhabiting Lake Tanganyika in East Africa, has the ability to recognize individuals and their social rank and can make social decisions to fight or flee based on the relative rank of conspecifics in the group (Grosenick et al., 2007). Interestingly, both medaka and cichlids exhibit the "face inversion effect," a phenomenon where recognition ability decreases when faces are inverted (Wang and Takeuchi, 2017; Kawasaka et al., 2019). This effect is well-known in humans (Valentine, 1988), where it is thought that inverted faces activate not only face-specific recognition areas but also object recognition areas in the brain, leading to decreased face recognition ability (Matsuyoshi et al., 2015). The observation of this effect in medaka and cichlids suggests the possibility of face-specific recognition areas (face regions) in the brains of these fish species.

4. Conventional neuroscience approach has revealed the molecular basis of social behaviors in Japanese medaka

We identifyed of neurons involved in familiarity-dependent mate preference through a combination of gene manipulation and electrophysiological approaches (Okuyama et al., 2014). These studies have revealed that GnRH3 (Gonadotropin-Releasing Hormone 3) neurons play a crucial role in activating mate preference for visually familiarized males. Medaka possess three gnrh paralogous genes (*gnrh1*, *gnrh2*, and *gnrh3*). GnRH3, encoded by *gnrh3*, is a peptide consisting of 10 amino

acids, primarily expressed in the extra-hypothalamic GnRH neurons (Abe and Oka, 2011). The cell bodies of GnRH3 neurons are located in the terminal nerve, with their axons projecting widely throughout the brain, except for the pituitary gland (Yamamoto et al., 1995). These neurons produce and release the GnRH3 neuropeptide, modulating activity across extensive brain regions. Interestingly, the GnRH3 neurons exhibit two functional modes based on their spontaneous neural activity. In the default state, the GnRH3 neurons show a low pacemaker frequency (2-3 Hz), which could suppress female receptivity to all males (inhibitory mode). Conversely, visual familiarization increases the pacemaker frequency (5-6 Hz), which might activate female receptivity towards familiar males (preference mode). The importance of the GnRH3 peptide has been demonstrated through gene knockout analysis. The gnrh3 mutant females lost their ability to prefer mates, accepting neither familiarized nor unfamiliar males. Furthermore, in these mutants, the pacemaker frequency failed to increase after visual familiarity, as typically observed in wild-type individuals. This result suggests that the GnRH3 peptide is essential for the expression of the preference mode. These findings indicate that the terminal nerve GnRH3 neurons play an important role in the neural mechanism of mate preference based on visual familiarization (Okuyama et al., 2014).

We also show that the oxytocin system plays a significant role in familiarity-dependent mate preference in medaka (Yokoi et al., 2020). Under normal laboratory conditions, female medaka prefer familiar males, while males tend to indiscriminately court females. However, behavioral analysis of mutants for genes encoding oxytocin (OT) and oxytocin receptor (OTRa) has revealed that these genes have sex-specific effects on their partner preference. Oxytocin signaling (OT and OTRa) is necessary for eliciting female preference for familiar males and is also involved in the indiscriminate selection by males. In ot or otra mutant females, visual familiarity no longer affects female receptiveness, resulting in the loss of partner preference for familiar males. Conversely, ot or otra mutant males display courtship more frequently towards familiar females than novel ones. The importance of the oxytocin system has also been confirmed in behavioral paradigms of male mate-guarding behavior in a triadic relationship (Yokoi et al., 2020). In these tests, two males and one female are placed in a small tank, with the males competing for the female. Typically, the dominant male exhibits "mate-guarding behavior," maintaining a dominant position near the female and preventing rival males from approaching (Yokoi et al., 2015, 2016, 2020). Interestingly, when presented with a novel female, ot/otra homozygous mutant males lose in male-male competition against heterozygous mutant males. This suggests a decrease in sexual motivation towards novel females in the ot/otra mutant males. On the other hand, with a familiar female, ot/otra homozygous mutants tend to dominate wild-type males in triadic relationships, suggesting that these mutants might exhibit excessive mate-guarding toward familiar females. These findings indicated that oxytocin system dysfunction in males leads to an excessive preference for familiar females. These discoveries present an exciting contrast with research results in mammals. Studies using monogamous rodents (prairie voles) have shown that OT signaling promotes attachment behavior towards familiar partners in both females and males (Walum and Young, 2018). In monogamous species, the differences in partner preference between males and females tend to diminish. Both members of the pair typically share responsibilities such as resource protection, offspring rearing, and providing social support to each other. In contrast, medaka are not monogamous and show strong sex differences in reproductive strategies (Yokoi et al., 2020; Daimon et al., 2022), which may have given rise to the sex differences in OT function in medaka fish. Our study also demonstrates that OT function has a conserved role in influencing partner preference based on familiarity recognition from fish to mammals. These findings provide important insights into the evolutionary conservation and diversity of neuroendocrine mechanisms of social cognition and mating behavior.

5. Variety of Japanese wild medaka local populations

Wild-derived strains of medaka have been originally collected from more than 100 regions across East Asia and are currently maintained and provided as laboratory stocks by the NBRP medaka (Sasado et al., 2010) mainly at Utsunomiya University (https://shigen.nig.ac.jp/me daka/). Recently, genomes of all strains have been analyzed, and a database has been created. Previous studies demonstrated that Japanese medaka (O. latipes species complex) were divided into two genetically distinct genetic groups (Fig.2A, Sakaizumi et al., 1980, 1983; Katsumura et al., 2019): the Southern Japanese group (O. latipes) originated in northern Kyushu and spread mainly along the Pacific side, the Northern Japanese group (O. sakaizumii) originated in the Tajima-Tango region and spread along the Sea of Japan side to Aomori (Fig.2B, Katsumura et al., 2019). The advantage of having more than 100 local populations in Japan derived from common ancestors makes it easy to analyze how the original genomic information has changed due to environmental factors to produce phenotypes, given their phylogenetic relationships. (Fig. 2A). Several traits associated with adaptive evolution (environmental adaptation) were identified in these two groups (O. latipes and O. sakaizumii). For instance, individuals from the Northern group exhibit cold tolerance, maintaining stable embryonic heartbeats even at temperatures below 12-15°C. In contrast, some local populations of the Southern Japanese group lack cold tolerance and display arrhythmia-like symptoms under low-temperature conditions (Yoshimura et al., 2022). Furthermore, the northern group demonstrates higher reproductive capacity compared to the southern group. This trait has been suggested as a potential adaptation to high-latitude environments with shorter breeding seasons (Fujimoto et al., 2024). These findings highlight the diverse adaptive strategies evolved by different medaka populations/groups in response to their specific environmental challenges, providing valuable insights into the mechanisms of local adaptation in this species. However, comprehensive behavioral analysis of local populations has only just begun, and to date, behavioral comparisons have been made among a limited number of Japanese medaka strains derived from local populations. For example, a study comparing aggression between the northern and southern groups of Japanese medaka observed groups of four males within each strain (Kagawa, 2014). The results showed that dominant individuals from the southern group exhibited significantly higher frequencies of aggressive behavior compared to dominant individuals from the northern group. Furthermore, when northern and southern groups were paired, males from the southern population displayed significantly higher frequencies of specific types of aggressive behaviors. These experimental results demonstrated clear behavioral differences between the two groups. In addition, one study revealed differences in startle response characteristics among medaka inbred strains (Tsuboko et al., 2014). The startle response to a visual stimulus (light extinction) was compared among four inbred strains (HNI-I, HNI-II, HO5, Hd-rR-II1). HNI-I and HNI-II are inbred strains derived from a wild population in Niigata Prefecture and belong to the northern Japanese group, while HO5 and Hd-rR-II1 are inbred strains belonging to the southern group. Notably, significant differences were observed between the HNI-II and Hd-rR-II1 strains. The HNI-II strain showed high sensitivity and little habituation to repeated light stimuli. In contrast, the Hd-rR-II1 strain exhibited low sensitivity and rapid habituation. Quantitative trait locus (QTL) analysis using a F2 family between these two strains detected a major QTL associated with startle response habituation on linkage group 16 (maximum LOD score of 11.82). This study was the first example to quantify behavioral differences among medaka inbred strains and identify the genetic locus responsible for the differences (Tsuboko et al., 2014). However, the identification of the responsible genes and mutations and the elucidation of the adaptive significance of behavioral diversity have not yet been accomplished.

6. Evolutionary and genetic studies using medaka-related species in the Indonesian Archipelago

Related species of Japanese medaka (the family Adrianichthyidae) are widely distributed in East and Southeast Asia. Interestingly, 23 of 40 described species in Adrianichthyidae are endemic to Sulawesi, Indonesia. Sulawesi is situated at the convergence of the Eurasian and Australian tectonic plates and represents a hotspot of geological activity (Spakman and Hall, 2010). The central region of this main island is characterized by a complex of ancient lakes, formed between the Pliocene and early Pleistocene epochs (5–1 Mya), whose lacustrine environments harbor an exceptional diversity of endemic medaka species (Mokodongan and Yamahira, 2015). This system presents a compelling model for investigating the mechanisms of adaptation and speciation in freshwater habitats. Recent works have yielded significant insights into the phenotypic and genetic characteristics of these medaka-related species (Fig.3).

A notable example is Oryzias woworae, a Sulawesian species endemic to Muna island located in Southeast Sulawesi, which exhibits pronounced sexual dimorphism (Fig.3C). The males possess red pectoral fins, a trait hypothesized to have evolved through sexual selection (Sumarto et al., 2021). Specifically, it is posited that female preference for this conspicuous trait has acted as a primary evolutionary driver, resulting in the elaboration of male-specific red coloration in the pectoral fins. We successfully identified *csf1* as a gene responsible for the male-specific pigmentation in O. woworae, employing a combination of linkage mapping and CRISPR-Cas9 mediated genome editing. Moreover, behavioral assays using csf1 mutants suggest that this sexually dimorphic coloration plays a role in female mate choice. This study is a rare example that partially reproduced species-specific phenotypic differences using gene editing methods, as shown in Fig. 1b (Ansai et al., 2021). The O. woworae system will present an opportunity to address several fundamental questions both in evolutionary ecology and neuroscience:

- 1. What are the underlying neural mechanisms mediating female visual preference of male secondary sexual characteristics?
- 2. How did genomic alterations between closely related species contribute to the evolution of these neural circuits for female mate preference using visual cues?
- 3. How did the temporal and mechanistic framework of these genomic changes drive evolution of sexually dimorphic coloration within the *O. woworae* lineage?

These inquiries intersect with long-standing fundamental questions in both neuroscience and evolutionary ecology, necessitating an integrative approach that combines methodologies from both disciplines.

Another intriguing system is Oryzias celebensis, an endemic species to Southwest Sulawesi (Fig.3D). This species exhibits rapid, contextdependent body color change (blackening), similar to the chromatic plasticity in chameleons and cephalopods. While such color changes often serve a dual function of crypsis and social signaling in other taxa (Stuart-Fox and Moussalli, 2008), we have recently reported that the blackening in O. celebensis males functions both as camouflage and as threat signals in agonistic interactions (Ueda et al., 2024). Specifically, males exhibiting dark coloration may suppress aggressive behavior in conspecific rivals. This phenomenon aligns with the concept of "sign stimuli" or "releasing stimuli" introduced by Nikolaas Tinbergen, a pioneer in ethology. While the neural and molecular underpinnings of chemosensory and auditory social cues have been extensively studied, the mechanisms mediating visual signal perception and its translation into behavioral outputs remain largely unexplored, particularly from a molecular genetic perspective. The O. celebensis therefore presents an excellent model system to investigate several critical questions in behavioral neuroscience and evolutionary biology:

- 1. What are the neural circuits and molecular pathways involved in the visual perception of "sign stimuli (conspecific color signals)" and the subsequent modulation of aggressive behavior?
- 2. How were neural circuits for camouflage modified to be utilized as social signals? It is predicted that social signals evolved as a cooption of camouflage because blackening through camouflage is widely observed in teleost fishes.
- 3. What genomic changes underlie the evolution of the neural systems?
- 4. How can we reconstruct the evolutionary trajectory of these genomic/neural changes within the evolutionary trajectory of the *O. celebensis* lineage?

By leveraging these unique medaka systems and employing cuttingedge genomic, neurobiological, and behavioral approaches, we stand to gain significant insights into the mechanistic basis of visual communication in fish and the evolutionary processes shaping complex behavioral traits in vertebrates.

7. Neuroscientific approaches to investigate the neural mechanisms of behavioral diversification in medaka fishes

How have the behavioral differences observed among medaka strains and species arisen through the process of brain evolution? These differences may be caused by changes in the number of neurons, changes in the relative proportions of cell types that make up these neurons, or the emergence of new cell types. Single-cell RNA sequencing (scRNAseq) has emerged as a powerful tool to address this question (Jourjine and Hoekstra, 2021). scRNA-seq enables the quantification of gene expression profiles at the single-cell level, allowing for the definition of cell types and quantitative comparison of these cell types among brain regions and/or species. This technology has opened up avenues for understanding how the evolution of cell types contributes to natural variations in neural circuits underlying behavior (Fig. 4). To investigate the evolution of cerebellar nuclei, single-cell comparative

transcriptomics among humans, mice, and chickens provided evidence of prototypical cerebellar nuclei that diversified through replication of specific cell types and subsequent divergence (Kebschull et al., 2020). In 2022, a series of four new studies published in Science revealed the existence of conserved neural types in the telencephalon across vertebrates based on interspecies comparisons of scRNA-seq profiles among mammalian, amphibian, and reptile brains (Hain et al., 2022; Woych et al., 2022; Lust et al., 2022; Wei et al., 2022). Furthermore, in 2023, scRNA-seq profiles were obtained in teleost fish telencephalon of zebrafish (Pandey et al., 2023) and cichlids (Johnson et al., 2023), revealing the existence of conserved neural types between fish and mammals. However, these studies compared evolutionarily distant species, and it remains unclear how different cell types among species can be involved in species-specific behaviors. To address this challenge, it would be effective to conduct scRNA-seq and interspecies comparisons on the brains of medaka species that have diverged relatively recently (other closely related medaka species and Japanese medaka). By using medaka species as the ingroup and zebrafish and/or cichlids as the outgroup, it may be possible to obtain more detailed evolutionary insights by comparing the proportions and types of cell types among different strains and species of medaka.

8. Technological advancements for medaka evolutionary social neuroscience

To further advance comparative studies among medaka species and strains, comprehensive behavioral analysis using identical behavioral paradigms is necessary. Recent advancements in machine learning can be applied to automated behavioral analysis, utilizing packages that quantify animal position and posture at high resolution. For example, DeepLabCut (Mathis et al., 2018) and you only look once (YOLO) systems have become available in medaka fish. The YOLO system, in particular, can extract animal behavior types and postures in real-time, potentially contributing significantly to the automation of behavioral



Fig. 4. Detection and functional analysis of species/strain-specific cell types in medaka fishes (A) Integration of single-cell transcriptome profiles of brains from different species (strains). Determination of cell types common to medaka species and those specific to certain species (strains), and identification of marker genes for each cell type. Using the promoter activity of marker genes, functional analysis of cell types using strains that express channelrhodopsin, calcium indicators, etc. (B) An example of optogenetics in adult medaka. Pectoral fin movement was induced by optogenetic activation in adult medaka knock-in line inserting a channelrhodopsin expression cassette into the upstream of *isl1* locus. By irradiating channelrhodopsin-expressing region with a laser, the activity of specific neurons can be non-invasively controlled. (C) An example of calcium imaging analysis in larval medaka. Brain activity was monitored using a transgenic line expressing a calcium indicator throughout the brain. Calcium signals, indicated by white arrowheads, were detected in the optic tectum in response to the movement of paramecia shown by the dotted ellipse.

experimental systems (Yamanouchi et al., 2023). In addition, based on 3D tracking, multiple body points (posture) across multiple individuals in fish groups can be tracked with individual identity (O'Shaughnessy et al., 2023).

Genetic tools for functional analysis of neurons and neural circuits have been also rapidly evolving in recent years. These include various channelrhodopsins for manipulating neural activity (Deisseroth and Hegemann, 2017), genetically encoded calcium indicators (Grienberger and Konnerth, 2012) for monitoring neural activity, and binary transgene expression systems (e.g. GAL4 enhancer trap lines) for analyzing specific cell types (Asakawa et al., 2008). In medaka, we have developed a technique for non-invasive manipulation of adult brains using optogenetics (Fig. 4B Seki et al., 2023) and have succeeded in calcium imaging techniques for larval fish (Fig. 4C). Furthermore, we also confirmed the effectiveness of the Tet system for labeling specific neurons for functional analysis in medaka embryo and adults (Hosoya et al., 2021; Kayo et al., 2024), similar to the GAL4 enhancer/gene trap in zebrafish and fruit fly, which will rapidly promote the development of tools for studying neurons and circuits of species/strain-specific cell types identified by scRNA-seq analysis. Importantly, recent advances in targeted gene knock-in techniques in medaka (Murakami et al., 2017; Watakabe et al., 2018; Seleit et al., 2021; Kayo et al., 2024) can further facilitate labeling of the specific cell types without labor works required for promoter identification and cloning.

9. Future prospects and challenges

The first step in understanding the causes of behavioral diversity among medaka fishes is to accumulate case studies on species (strain)specific behaviors. However, the ultimate goal of evolutionary neuroscience is to understand why certain behaviors or neural mechanisms are expressed in some lineages or species and not in others. To achieve this goal, it is necessary to identify general principles that go beyond individual case studies. Recently, we conducted a comprehensive comparison of traits (specifically gut length) in wild populations of Japanese medaka and successfully answered the important question in evolutionary biology: "How does phenotypic plasticity contribute to adaptive evolution?" Although most Japanese medaka local populations show seasonal phenotypic plasticity in gut length (longer in summer, shorter in winter), some local populations in the northern Japanese group show a fixed phenotype of gut length (Katsumura et al., 2020). We successfully identified candidate genes involved in phenotypic plasticity and genetic fixation by combining genetic and epigenetic approaches such as GWAS, genome-wide methylation analysis, and gene expression analysis with medaka genomic information and phenotypes. Furthermore, we experimentally verified that changes (deletions) in the promoter region of the gene involved in phenotypic plasticity give rise to new phenotypes using gene editing techniques. In this way, by combining evolutionary approaches and genetic manipulation in wild medaka populations, it has enabled direct testing of general principles in evolutionary biology. In the field of behavioral biology, several research groups have just begun comprehensive behavioral comparisons between medaka local groups, and it is expected that the molecular genetic basis for behavioral diversities among local populations will be elucidated using the similar research strategy.

In cichlid fishes, identifying the neurological and molecular causes of behavioral differences between lineages and species has revealed examples of behavioral adaptations (e.g., Campbell et al., 2019) and new mechanisms that may underlie these adaptations (e.g., Okobi et al., 2019). Cichlid fishes, in particular, exhibit remarkable species richness and phenotypic diversity in morphology, behavior, and body color. Moreover, over 2000 species have diversified based on ecological niches within lakes, often evolving in parallel within and across radiating lineages. Cichlids have a history of adaptive radiation from a common ancestor within limited geographical areas. Consequently, it is postulated that diversification has progressed through ecological speciation

under divergent selection pressures, despite relatively low genetic variation. These characteristics have made cichlids a prominent model system for studying genomic diversification driven by natural and sexual selection. Recently, Sommer-Trembo and colleagues elucidated the genetic basis of exploratory behavior in the adaptive radiation of cichlid fish species in Lake Tanganyika (Sommer-Trembo et al., 2024). Using a standardized open-field test, they quantified exploratory behavior in 702 individuals from 57 species and then identified 1199 genetic variants strongly linked to exploratory tendencies by a GWAS approach. Notably, a single nucleotide polymorphism (SNP) upstream of the cacng5b gene, which regulates AMPA-type glutamate receptors, showed an almost perfect correlation with species-specific exploratory tendencies and the cacng5b expression level. They validated that promoter mutations induced by CRISPR-Cas9 system could mimic the alteration of exploratory behavior. This study provides valuable experimental evidence that behavioral traits are involved in niche adaptation during large-scale adaptive radiation.

In medaka fish species, advances in genetics and genomics approaches will enable us to identify genes and mutations responsible for the behavioral diversity. In medaka, forward genetics approaches such as QTL mapping (Tsuboko et al., 2014; Ansai et al., 2021) and genome-wide association studies (GWAS) (Katsumura et al., 2020) are effective for describing the genetic architecture of trait variations and are gaining renewed attention due to increasingly affordable genome sequencing and genotyping methods. These approaches have been successfully used in various species to identify the genetic basis of morphological variations and are now applied to behavioral variations (Niepoth and Bendesky, 2020). Therefore it is anticipated that utilizing wild populations of Japanese medaka and the Indonesian medaka-related species will lead to breakthroughs in understanding the genetic foundations of behavioral differences between species, similar to the recent findings in cichlids. This advancement in medaka evolutionary social neuroscience is expected to provide deep insights into the diversity of social behavior in organisms.

10. Conclusion

The study of medaka fishes as a model for evolutionary social neuroscience offers a unique opportunity to bridge the gap between neuroscience and evolutionary ecology. By leveraging the rich genetic resources, comprehensive genomic information, and the ease of laboratory manipulation, researchers can gain unprecedented insights into the proximate and ultimate causes of behavioral diversities. The exploration of visual-based mate preference and the application of cutting-edge technologies such as scRNA-seq and machine learningbased behavioral analysis are opening new avenues for understanding the evolution of social cognition and mating strategy. As we continue to unravel the complexities of behavioral diversity in medaka and its relatives, we not only enhance our understanding of fish behavior but also can contribute to the broader field of neuroscience. The principles and mechanisms that could be discovered in medaka may have far-reaching implications for our understanding of social cognition and behavior across the animal kingdom. The field of medaka evolutionary social neuroscience shows promise and will contribute to our understanding of brain evolution and function. As we move forward, the integration of diverse approaches-from molecular genetics to behavioral ecology—will be crucial in realizing the full potential of this promising field.

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CRediT authorship contribution statement

Takafumi Katsumura: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing - original draft, Writing - review & editing. Takahide Seki: Data curation, Investigation, Methodology, Visualization. Saori Yokoi: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. Towako Hiraki-Kajiyama: Conceptualization, Data curation, Investigation, Project administration, Supervision, Writing - original draft, Writing - review & editing. Ryutaro Ueda: Data curation, Investigation, Visualization. Hideaki Takeuchi: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing - original draft, Writing - review & editing. Satoshi Ansai: Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

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