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Latent gene network expression underlies partial re-evolution of a polyphenic trait in the worker caste of ants

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eLife Assessment

This **important** study explores whether complex structures that are lost during evolution can re-evolve, which is a long-standing debate in evolutionary and developmental biology. The authors demonstrate that re-evolution can occur if the gene regulatory network that underlies the development of complex traits is maintained. The evidence supporting its conclusions is **convincing** and the work will be of interest to those studying the evolution and development of complex traits.

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Abstract

Polyphenisms—where alternative phenotypes develop from a single genome in response to environmental cues—are not only widespread in nature, but also occur at multiple levels of biological organization, from cells to individuals to societies. Polyphenism is thought to promote phenotypic diversification through the gain, loss, and re-evolution of alternative phenotypes. After the origin of a polyphenism, one of the alternative phenotypes often retains the developmental capacity to produce the ancestral trait, thereby permitting the other to evolve rapidly. Yet, little is known about the developmental processes underlying the re-evolution of polyphenic traits and how they may produce phenotypic diversification. Here we address this question by focusing on the caste polyphenism in ant societies, which produces a winged queen caste and a wingless worker caste in a single colony in response to environmental cues. We show, in a hyperdiverse group of ants, that a caste-specific trait called the ocelli (3 simple eyes on the dorsal head) is always present across queen castes but was lost and partially re-evolved multiple times, giving rise to novel patterns (1 ocelli) in the worker castes. Surprisingly, we discovered that a hidden (latent) expression of the ocelli gene regulatory network in worker castes that lost ocelli underlies the partial re-evolution of ocelli in this group. We therefore propose that latent developmental potentials may generally persist across polyphenic systems, including ant castes, and may facilitate the partial re-evolution of novel phenotypic patterns.

Introduction

Polyphenism is a form of developmental plasticity where alternative phenotypes develop from a single genome in response to environmental cues (Nijhout, 2003).It is phylogenetically widespread feature of plants and animals that has evolved at different levels of biological organization (Hanna et al., 2024; West-Eberhard, 1989, 2003). For example, at the population level, the mouth form polyphenism in nematode worms produces alternative big tooth (omnivorous) or small tooth (bactivorous) mouth forms that develop in response to pheromones, crowding, salt concentration, temperature, and culturing substrate (Bento et al., 2010; Bose et al., 2012; Ragsdale et al., 2013; Werner et al., 2018). At the colony-level, caste polyphenism in eusocial insects produces morphologically differentiated queen and worker castes that develop in response to temperature and nutrition (Chandra et al., 2018; Evans & Wheeler, 2001; Korb, 2025; A. Rajakumar et al., 2024). And finally, at the cellular-level, polyphenism occurs within a multicellular individual, where a single genome gives rise to differentiated cell-types during development, such as between germline and somatic cells, in response to internal cues like morphogen gradients (Brunet & King, 2017; Davison & Michod, 2021; Devlin et al., 2023). Polyphenism has been proposed to promote, at the macroevolutionary scale, phenotypic diversification through the gain, loss, and re-evolution of alternative phenotypes (West-Eberhard, 2003). This is based on the idea that, once a polyphenic trait originates, one of the alternative morphs retains the capacity to produce the trait in the genome while the other is freer to evolve. This hypothesis has received support from a comparative study of mouth form polyphenism across 90 species of nematode worms showing a phylogenetic association between the gain and loss of alternative mouth form phenotypes and the phenotypic diversification of mouth parts (Susoy et al., 2015). Another supporting example is fat synthesis in parasitic wasps, which revealed an association between developmental plasticity and the loss and subsequent re-evolution of fat synthesis in one species (Peters et al., 2017; Visser et al., 2010, 2021). Yet, the underlying developmental and genetic processes facilitating the gain, loss and re-evolution of polyphenic traits remain poorly understood. (Forni et al., 2026; Sommer, 2020; West-Eberhard, 2003).

Here we address this question by focusing on caste polyphenism in the eusocial colonies of ants, which consists of a morphological division of labor between a winged reproductive queen caste and wingless non-reproductive worker caste in almost all 16,962 valid described ant species (AntWeb, 2026; Ward, 2014). The differential expression of polyphenic traits, such as wings, that develop in queens but not workers, are called "caste-specific" traits (Miura, 2005). It has been shown that polyphenic traits, including caste polyphenism in ants, are produced during development by the differential expression of highly conserved gene regulatory networks (GRN) in response to environmental cues (Abouheif & Wray, 2002; Béhague et al., 2018; Casasa et al., 2021; Davidson et al., 2023; Lenuzzi et al., 2023; A. Rajakumar et al., 2024; Vizueta et al., 2025). However, how the expression of these GRNs influences the evolution of caste-specific traits in ants remains unknown.

Here we focus on the ocelli, which are 3 small single-lens eyes on the dorsal head of most flying insects. Ocelli complement the function of the compound eyes by mediating orientation using polarised light and in the synchronization of daily activity (Buschbeck & Bok, 2023; Krapp, 2009). We investigate the evolution of ocelli in a hyperdiverse subfamily of ants (Formicinae), where they are universally present in the winged reproductive caste (queens and males) as 3 large ocellus that aid in mating flights and dispersal (Moser et al., 2004; Narendra et al., 2016). In contrast, ocelli in the wingless worker caste are evolutionarily labile, showing dramatic variation across species in the presence / absence or number of ocelli in the worker caste (Johnson & Rutowski, 2022; Narendra et al., 2016; Narendra & Ribi, 2017; Schwarz et al., 2011) (Figure 1A). In some species, adult workers completely lack ocelli, such as in *Camponotus floridanus*, while in others they are present and vary in number—there are species whose adult workers have all 3 ocelli or just 1 single ocellus, and these can be present in all or in only in a subset of workers (Figure 1A). For example, workers in *Cataglyphis bicolor* have all three ocelli, which function in light sensing and navigation, acting as a celestial compass that provides crucial directional information (Fent & Wehner, 1985) (Figure 1A). In contrast, all workers of *Polyrachis bihamata* have just a single medial ocellus (Hung, 1967) (Figure 1A), and in workers of *Dinomyrmex gigas*, a single medial ocellus evolved only in a subset of individuals in the worker caste called 'soldiers' (or major workers) with large heads, but are absent in other individuals called 'minor workers' with small heads (AntWeb, 2026) (Figure 1A). How this dramatic variation in ocelli in the worker caste of formicine ants has evolved remains poorly understood.

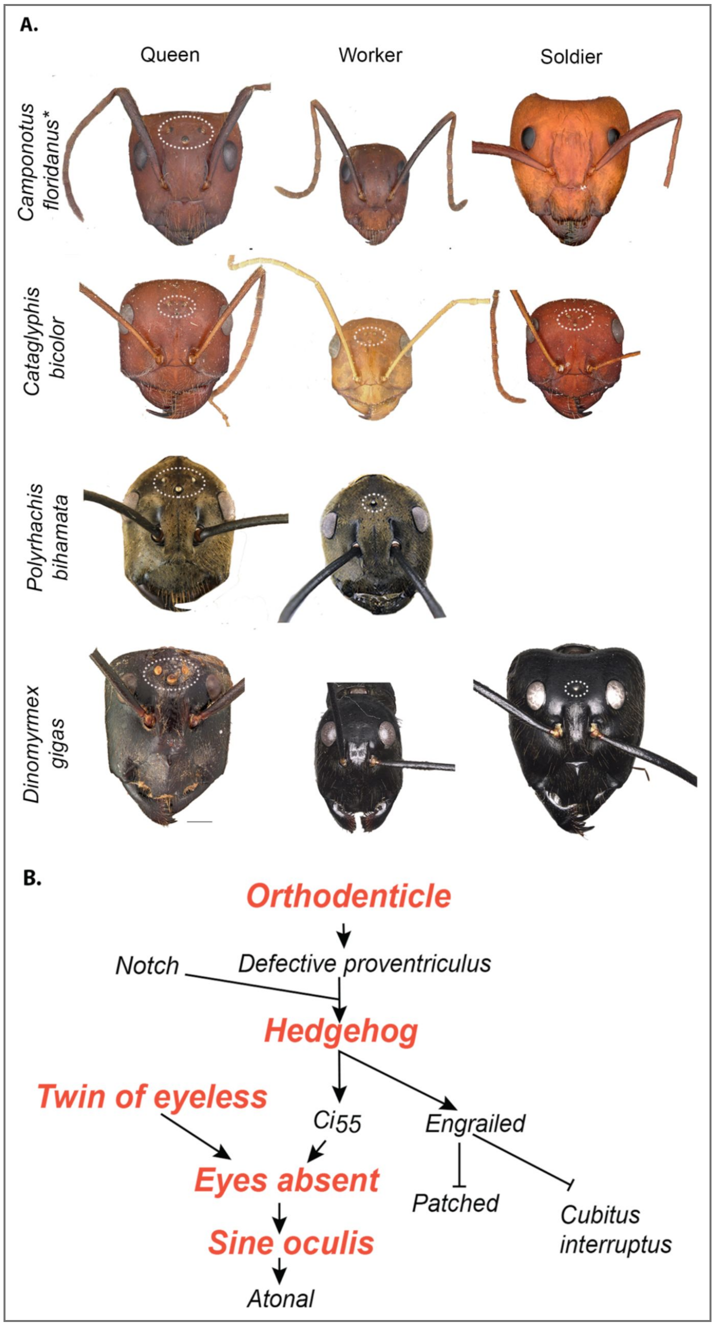


Figure 1. **The presence and absence of ocelli in queens and workers across ants.**

(A) Ocelli develop in the winged reproductive castes across species of formicinae ants exemplified by *Camponotus floridanus*, *Cataglyphis bicolor, Polyrachis bihamata* and *Dinomyrmex gigas* (white dashed circles). Ocelli in the worker caste of formicine ants (white dashed circles) are evolutionarily labile, showing no ocelli (*Camponotus floridanus*), all 3 ocelli in all individuals of the worker caste (*Cataglyphis bicolor*) or only 1 ocellus in only the soldiers (*Polyrachis bihamata* and *Dinomyrmex gigas*). (B) Schematic representation of the ocelli GRN in *Drosophila melanogaster* adapted from Jean-Guillaume & Kumar (2022)(Jean-Guillaume & Kumar, 2022). The genes investigated in this study are highlighted in orange. Arrowheads indicate activation, and bars indicate repression. Queens are scaled to 1mm, and workers and soldiers are scaled according to the queen of each species. Asterisks indicate that *C. floridanus* was used for gene expression studies. Photos from Antweb (AntWeb, 2026)

This evolutionary lability of ocelli across the worker castes of formicine ants also provides an opportunity to understand how the GRN underlying development of ocelli influenced the evolution of this caste-specific trait. Ants are holometabolous insects, in which adult body parts develop from imaginal discs, semi-independent clusters of cells in the larvae (Held, 2002; Koch & Abouheif, 2019). In the fruit fly *Drosophila melanogaster*, ocelli develop from the eye-antenna imaginal disc located at the ventral region of the head capsule. The eye-antenna imaginal disc also gives rise to the head capsule, eye, antenna, and maxillary palps (Held, 2002). *D. melanogaster* is the only insect where the GRN underlying ocelli development has been well characterized at the third larval stage (Blanco et al., 2009; Domínguez-Cejudo & Casares, 2015; Sabat et al., 2017) (Figure 1B). The gene *orthodenticle* (*otd*) (formerly known as *ocelli-less*) is a selector gene that is necessary for specifying the ocellar region in the developing head capsule. *otd* expression in the ocellar domain, together with other genes like *hedgehog* (*hh*), initiates the development of the ocellar region and the three ocelli (two lateral ocellus and one medial ocellus) in the eye-antennal disc. The activation of these genes regulates the expression of downstream genes, such as *otd* regulating the expression of *defective proventriculus* (*dve)* (Blanco et al., 2009; Jean-Guillaume & Kumar, 2022; Yorimitsu et al., 2011), whereas *hedgehog* (*hh*) activates a portion of the retinal determination network, such as *eyes absent* (*eya*), *twin of eyeless* (*toy*), *sine oculis* (*so),* and *atonal* (*ato*). These retinal determination genes have been shown to be regulated by independent regulatory enhancers from the compound eye (Blanco et al., 2009, 2010; Jean-Guillaume & Kumar, 2022) (Figure 1B). Because the eye-antennae disc and the ocelli GRN have only been well characterized in *D. melanogaster*, it remains unknown whether they are conserved in ants.

To understand how the GRN underlying ocelli development may have influenced the evolution of this caste-specific trait, we first inferred the evolutionary history of ocelli in adult workers across the Formicinae using ancestral state reconstruction. We then characterized the eye-antennae disc in ants using three genes, *eyeless* (*ey*), *distal-less* (*dll*), and *otd-1*, which are known to mark the eyes (*ey*), the antenna (*dll*), and the head capsule and ocelli (*otd-1*). This characterization allowed us to investigate the expression of five key genes in the ocelli GRN, *otd-1*, *hh*, *toy*, *eya*, and *so*, during development of the winged and wingless castes across two formicine species.

Results

Partial reversion to a single ocellus occurs 3 times independently within the tribe Camponotini (Formicinae)

We first performed an ancestral state reconstruction to infer the evolutionary history of worker ocelli across the subfamily Formicinae. Our ancestral state reconstruction inferred a single re-gain of worker ocelli at the base or early within the Formicinae (Figure 2). Subsequent to the gain of ocelli early in the evolution of the Formicinae, our analysis inferred a single, well-supported, loss of worker ocelli at the base of the tribe Camponotini (Figure 2). Following this single loss, we inferred three (well supported) independent and partial re-evolution to a single medial ocellus in three different genera: *Camponotus gibbinotus*, *Polyrachis bihamata*, and *Dinomyrmex gigas* (Figure 2). In *P. bihamata*, the single ocellus occurs in all workers in the colony, whereas in *C. gibbinotus* and *D. gigas* the single ocellus occurs only in the large-headed soldiers (Figure 1). We therefore investigated the developmental role of the ocelli GRN underlying these independent partial reversions to a single medial ocellus in this tribe of ants.

A fate map characterizing the development of the head capsule, antennae, eyes, and ocelli within the eye-antenna disc in ants

To understand whether the GRN underlying the development of ocelli influenced the reversions of this caste-specific trait in workers, we first had to characterize the development of the eye-antenna disc in ants. In *D. melanogaster*, the eye, antennae, maxillary palps, ocelli, and head capsule develop from the eye-antenna disc, which is segregated into regions marked by the expression of highly conserved developmental genes (Haynie & Bryant, 1986; Held, 2002). In the Florida

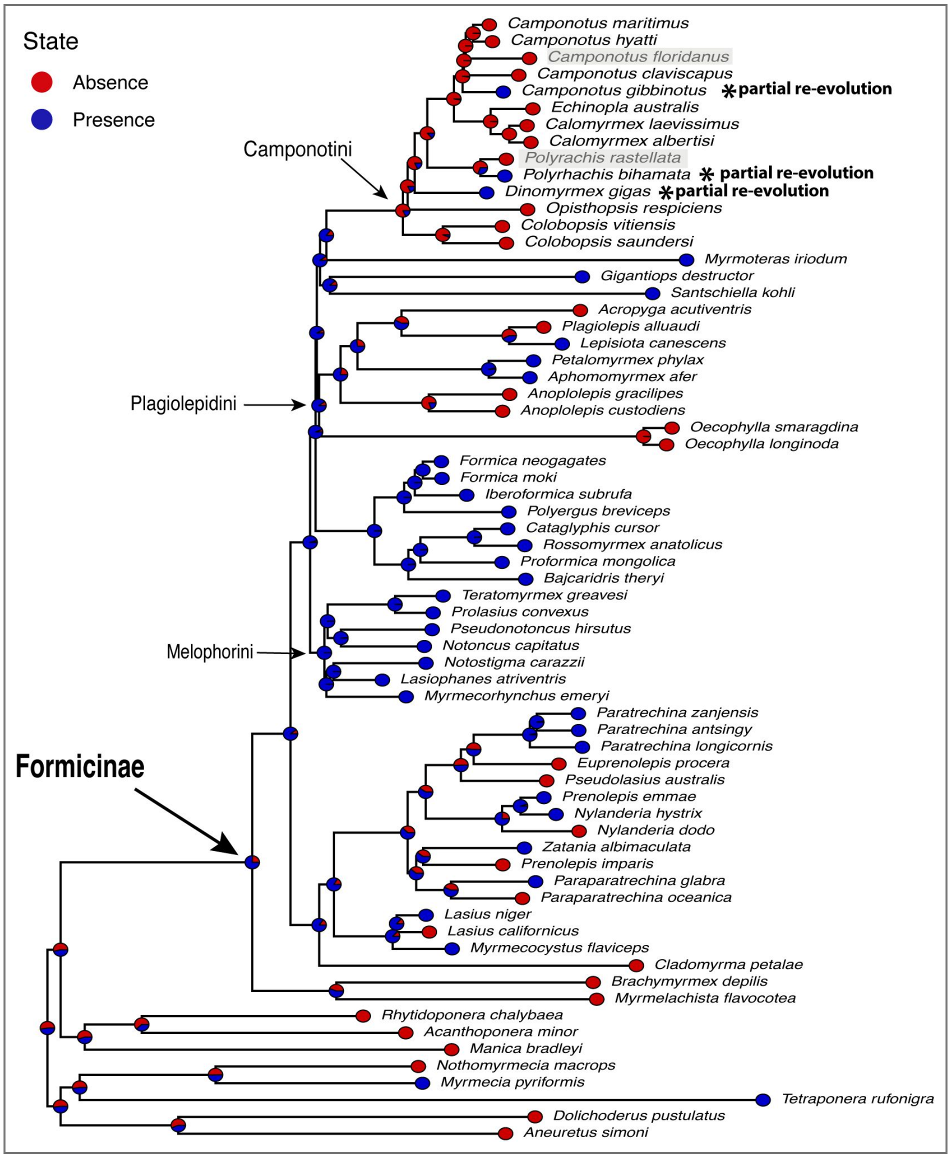


Figure 2. **Ancestral state reconstruction of ocelli reveals three well-supported reversions of ocelli in the worker caste in the tribe Camponotini.**

Maximum clade credibility tree of formicine ants from Blaimer et al.(Blaimer et al., 2015). Ancestral state reconstruction for the presence (blue-colored circles) and absence (red-colored circles) of ocelli based on stochastic character mapping. Each pie chart for the nodes represents the posterior probabilities, scaled by the weight of evidence for each model. Species used to analyze ocelli GRN expression are highlighted in gray, and the species that re-evolved one ocellus is indicated as partial re-evolution. Three tribes within the Formicinae are marked (Melophorini, Plagiolepidini, Lasiini, Myrmelachistini and Camponotini) by arrows.

carpenter ant *C. floridanus*, we found that, similar to *D. melanogaster*, expression of *otd-1* marks the precursor regions of the head capsule and ocelli, *ey* marks the precursor regions of the eyes, and *dll* marks the precursor regions of the antennae. During the first larval instar, expression of *otd-1* emerges primarily in the head capsule in middle part of the disc between the antenna and compound eye (Figure 3B.). In contrast, *dll* and *eya* expression delineate the precursor regions of the antenna and eye (Figure 3C, D). During the second and third larval instar, *otd-1* is expressed in the developing head capsule and ocelli in the medial region of the disc (Figure 3 G, L), while *dll* is confined to the antenna and *ey* to the compound eye region (Figure 3H, I, M, N). Finally, during the fourth (final) larval instar, a developmental threshold mediated by juvenile hormone acts as switch point to determine whether larvae will develop either into a minor worker or soldier (MacMillan et al., 2025). Once larvae have been determined, expression patterns of *otd-1*, *dll*, and *ey* in worker-destined larvae (Figure 3 P to T) or soldier-destined larvae (Figure 3 U to Y) remain expressed in the same regions as in the second and third instars (Figure 3Q to T, V to Y). Together, our characterization shows that the eye-antennae disc and the regional identities within it, including the precursor region of the head capsule, ocelli, eyes, and antennae, are conserved in ants relative to *Drosophila*.

Expression of the ocelli GRN is conserved in winged reproductive castes but is latent in species whose adult workers completely lack ocelli

We next asked whether the ocelli GRN is conserved in the winged reproductive caste (males) relative to *Drosophila* and whether it is expressed in workers that entirely lack ocelli as adults. We address these questions using two species *C. floridanus* and *Polyrachis rastellata*. We chose these two species because *C. floridanus* is closely related to *C. gibbinotus* and *P. rastellata* is closely related to *P. bihamata*, which are two of the species that our ancestral state reconstruction inferred independent partial reversions to single medial ocellus in the worker caste (Figure 2). In the winged male caste of *C. floridanus*, we found that *otd-1*, *eya* and *so* are expressed where the 3 ocelli will develop, while *toy* and *hh* are expressed in the inter-ocellar region (the tissue that separates the three ocellus) (see white arrowheads in Figure 4A to B ' and 5A to 5C'). Because these genes are similarly expressed within the ocellar region within the eye-antennal disc of *D. melanogaster*, we infer that the ocelli GRN is conserved in the winged reproductive castes in ants.

Surprisingly, we discovered that the ocelli GRN remains latently expressed in minor worker- and soldier-destined larvae of *C. floridanus*, which completely lack ocelli as adults. In soldier-destined larvae, all five genes remain expressed in the ocellar region within the eye-antennal disc at the beginning of the last larval instar (Figure 4C, D and Figure 5D to F). By the end of this instar, *otd-1* and *hh* remain expressed (Figure 4C', D'), but *toy*, *eye*, and *so* are either down-regulated or absent relative to their expression in the compound eye region (Figure 5D' to F'). In minor worker-destined larvae, 3 of the 5 genes (*otd-1*, *hh, eya*) remain expressed in the ocellar region during the early part of the last larval instar (Figure 4E, F and Figure 5 H), whereas *toy* and *so* are expressed in the compound eye region but absent (interrupted) in the ocellar region (Figure 5G, I). Furthermore, in *P. rastellata*, whose worker caste is composed of similarly sized individuals with no subcastes, we found that *otd-1*, *eya*, and *so* remain expressed in the ocellar region within the eye-antennal disc during the early part of the last larval instar (Figure 6). Together, our results show that despite the absence of ocelli in adult workers for millions of years, the expression of the ocelli GRN remains latent during larval development.

Finally, we performed Scanning Electron Microscopy (SEM) in *C. floridanus* male, soldier, and minor worker pupae. In minor worker and soldier pupae, we discovered the existence of rudimentary ocelli that appear at the beginning of pupal development, continue to be elaborated, and then are eliminated before they molt into adult workers (Figure 7). These ocelli rudiments in are highly reduced relative to the fully functional ocelli found in male pupae. Finally, the pattern and timing of development of ocelli rudiments in the minor worker and soldier pupae coincides with the spatial expression and timing of interruption of the latent expression of the ocelli GRN. In soldier-destined larvae, ocelli GRN expression is interrupted later in development

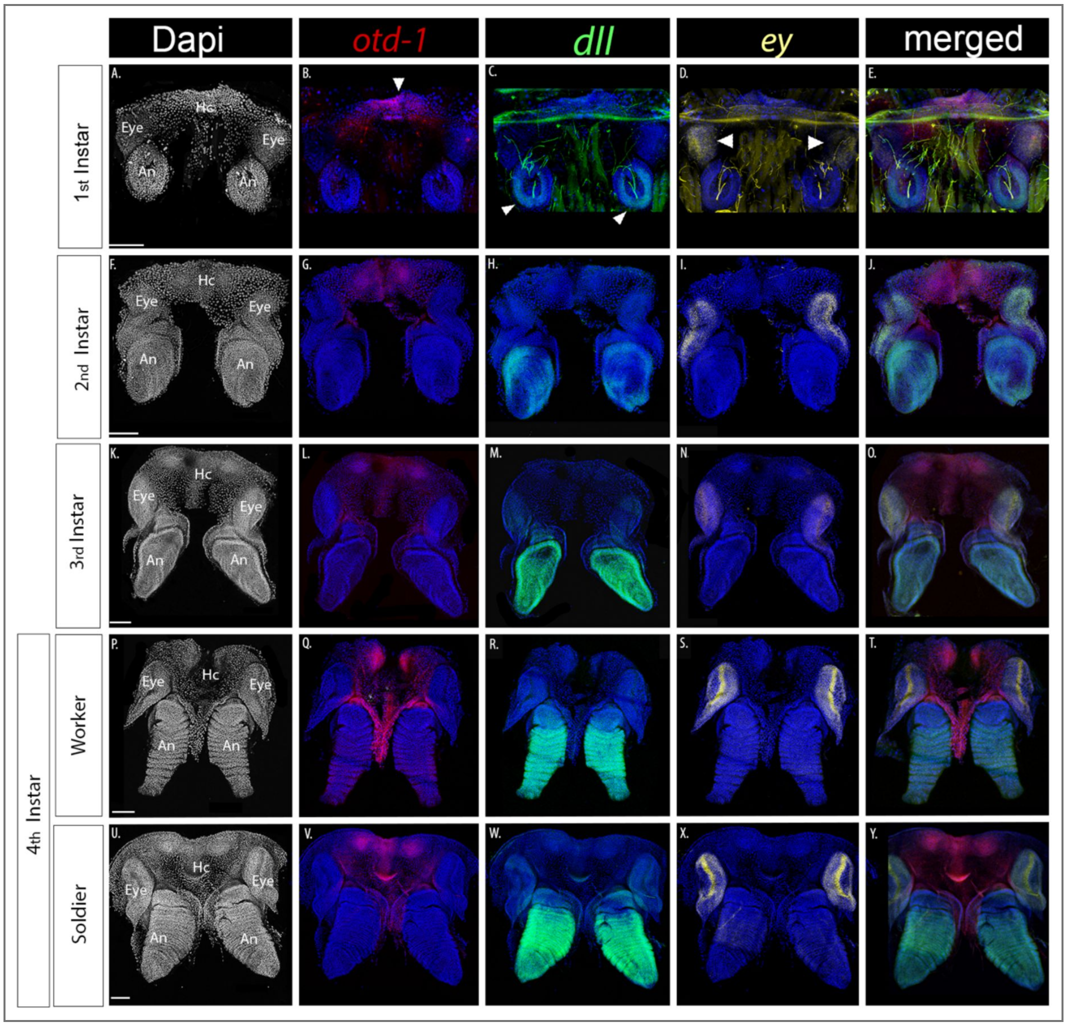


Figure 3. **Characterizing development of the eye-antenna imaginal disc in worker castes of *C. floridanus* using *orthodenticle-1 (otd-1), distal-less (dll)* and *eyeless (ey)* gene expression to mark the developing head capsule and ocelli, antenna, and eyes.**

Fluorescent images in panels A, F, K, P, U represent the development of the entire eye-antenna imaginal disc marked with the nuclear stain DAPI across all four larval stages, where the head capsule region is labelled as ‘Hc’, the antennal region is labelled as ‘An,’ and the eye region is labelled as ‘Eye.’ Panels B, G, L, Q, V represent the development of the head capsule (Hc) marked by the genes *orthodenticle-1 (otd-1* in magenta); Panels (C,H,M,R,W) represent the antennal region marked by *distal-less* (*dll*) in green color; and Panels (D,I,N,S,X) represent the eyes (Eye) is *eyeless (ey*) (yellow); Panels. (A-E) First instar. Note: the green or yellow staining outside of the structures highlighted by the white arrows in panels C, D and E is background noise. (F-J) second instar, (K-O) third instar, (P-T) fourth instar worker-destined larvae and, (U-Y). Fourth instar soldier-destined larvae. All images are to scale.

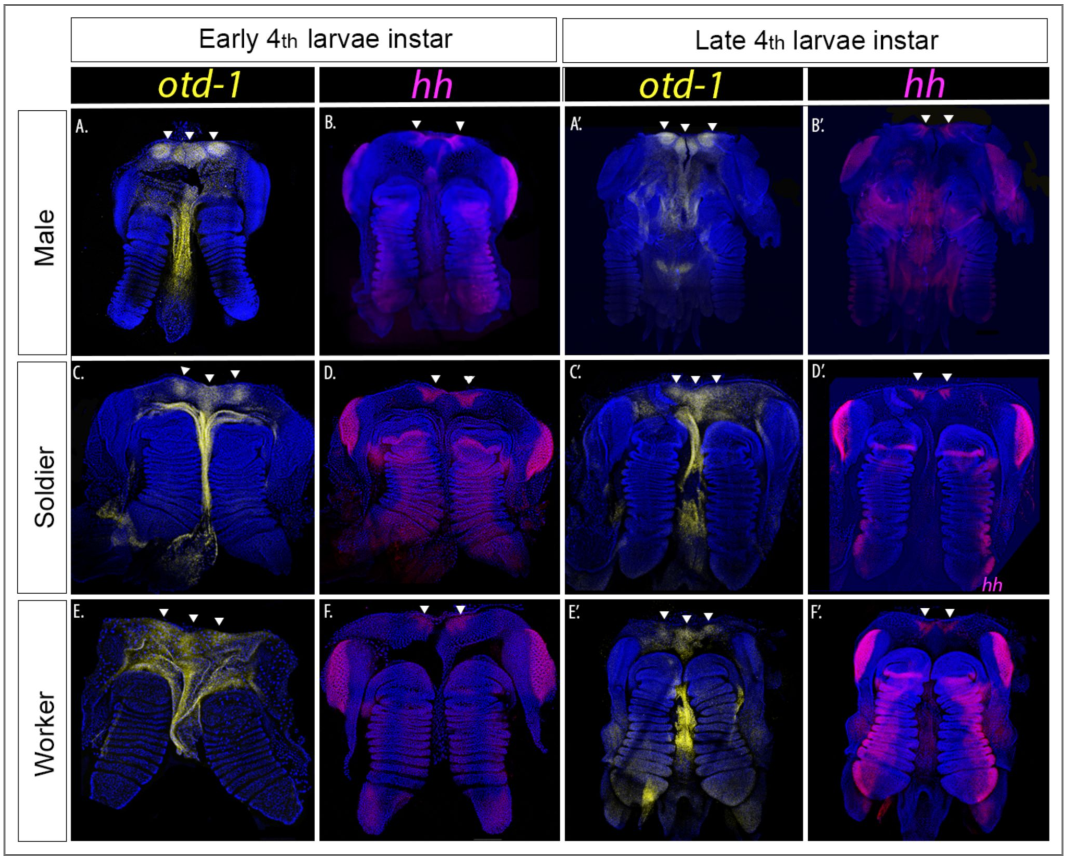


Figure 4. **Latent expression of *otd-1* and *hh* genes in the ocelli GRN in workers and soldiers of *C. floridanus* during the 4th larval instar.**

Expression of *orthodenticle-1(otd-1)* is yellow, and *hedgehog (hh)* is magenta. Early 4th instar; (A, B) males (C, D) soldiers and (E, F) workers. Late 4th instar: (A,’ B’) males (C’, D’) soldiers and (E’, F’) workers.

Figure 5. Latent Expression of *toy*, *eya*, and *so* within the ocelli GRN in the developing worker and soldiers of *C. floridanus*

Expression of *toy* (green), *eya* (yellow), and *so* (magenta). Early 4th instar;(A to C) males, (D to F) soldiers and (G to I) workers. Late 4th larvae stage;(A′ to C′) males, (D′ to F′), soldiers and (G′ to I′).

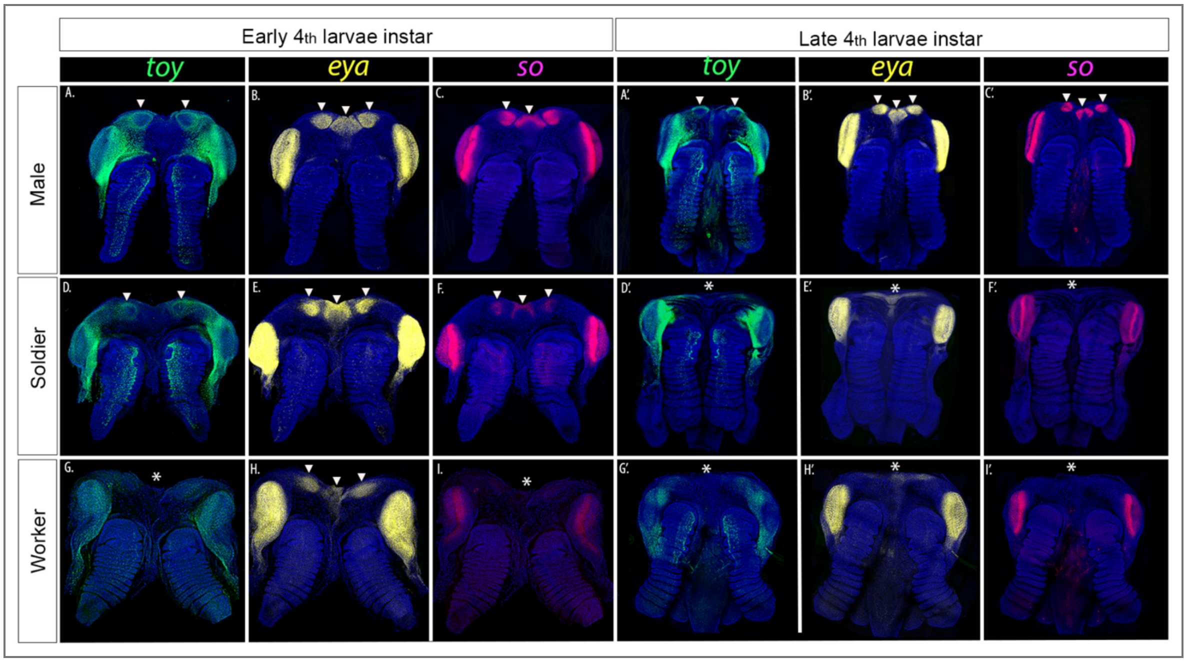
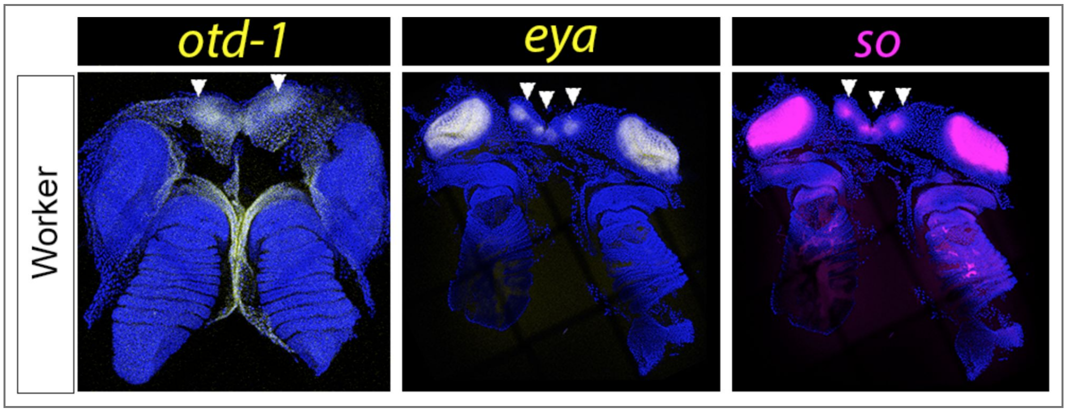


Figure 6. Latent expression of the ocelli GRN in the eye-antennal disc in worker larvae of *Polyrachis rastellata* at 4th larvae stage.

Expression of selected genes *otd-1* (yellow), *eya* (yellow), and *so* (magenta) at early 4th instar larvae.



than in minor worker-destined larvae, and consequently, the ocelli rudiments in soldier pupae continue to develop longer and are more elaborated relative to those in minor worker pupae (Figure 7B to D and F to H). Therefore, expression of the latent ocelli GRN in the eye-antennae disc results in the development of ocelli rudiments in worker pupae and are then eliminated in adult workers.

Discussion

Our developmental and evolutionary data provide evidence that the latent expression of genes in workers lacking ocelli as adults is part of a latently expressed ocelli GRN, which likely facilitated at least 3 independent evolutionary reversions of this trait in the worker caste of species within the Camponotini clade. The latent expression patterns of genes in the ocelli region of developing workers lacking ocelli as adults are the same as in males that will develop fully functional ocelli but are only interrupted late in larval development. Furthermore, the timing and pattern of these late interruptions coincide with the degree of development of rudimentary ocelli in minor worker and soldier pupae before they disappear in adults (Figure 7). This indicates that, although the expression of these genes is latent, they still retain the capacity to produce rudimentary ocelli in the pupal stage before they disappear in adults. And finally, although *eya*, *toy*, and *so*, are part of both the ocelli and compound eye GRNs in *Drosophila*, the ocelli GRN has its own distinct identity and the genes within this GRN have distinct regulatory elements and are selectively regulated (Jean-Guillaume & Kumar, 2022; Zimmerman et al., 2000). In ants, our data shows that the selector gene for compound eye development in insects (*eyeless* / *Pax-6*) is expressed in the compound eyes and not ocelli, and the selector gene for ocelli (*otd-1*) is expressed in the ocelli and not compound eyes. We further show that the latent expression of *toy*, *eye*, and *so* are downregulated or absent in the ocellar region, but at the same time, are strongly expressed in the regions of the compound eyes. Therefore, the mutually exclusive expression of the selector genes *eyeless* / *Pax-6* in the compound eyes and *otd-1* in ocelli suggests that, like in *Drosophila*, the compound eye and ocelli GRNs have distinct identities, ultimately leading to differential expression of downstream genes and production of different cell types; compound eyes are produced from multiple imaging forming facets, while ocelli are produced from a single lense (Buschbeck & Bok, 2023; Jean-Guillaume & Kumar, 2022; Mishra et al., 2021). Altogether, our data show that the expression of these genes in workers lacking ocelli as adults are part of a latently expressed ocelli GRN.

Several hypotheses may explain how the ocelli GRN came to be latently expressed and maintained in developing workers that lack ocelli as adults. Perhaps the most simplistic hypothesis proposes that the presence of functional ocelli in adult queen and male castes maintains the ocelli GRN intact in the genome by keeping it under positive natural selection. This hypothesis assumes that this, as a side consequence, leads to expression of the ocelli GRN in the worker castes lacking ocelli. However, caste determination between queens and workers occurs through the action of a developmental threshold or switch, where a continuous environmental cue is translated into discrete phenotypic outcomes (Abouheif, 2021; MacMillan et al., 2025; Qiu et al., 2022; A. Rajakumar et al., 2024; Schultner et al., 2023). Once caste determination has occurred, the genome is expressed differentially during the developmental trajectories of queens and workers (Abouheif, 2021; Abouheif & Wray, 2002; Barkdull & Moreau, 2023; Béhague et al., 2018; Chandra et al., 2018; Khila & Abouheif, 2010; Qiu et al., 2022; Vizueta et al., 2025). These trajectories are decoupled, and consequently, can evolve largely independently (Abouheif, 2021; Powell et al., 2020; Vong et al., 2025). The dramatic variation in the number, size, and presence/absence of ocelli in worker castes across the Formicinae (see Figure 1) supports the largely independent evolution of ocelli in workers from those in queens, which always develop 3 ocellus. Furthermore, we observe similar patterns of variation in the wings and ovaries between queen and worker castes across ant species (Cronin et al., 2013; Khila & Abouheif, 2010; Monnin & Peeters, 2008; R. Rajakumar et al., 2012, 2018). Therefore, while the presence of ocelli in males and queens maintains the ocelli GRN in the genome and creates a potential for

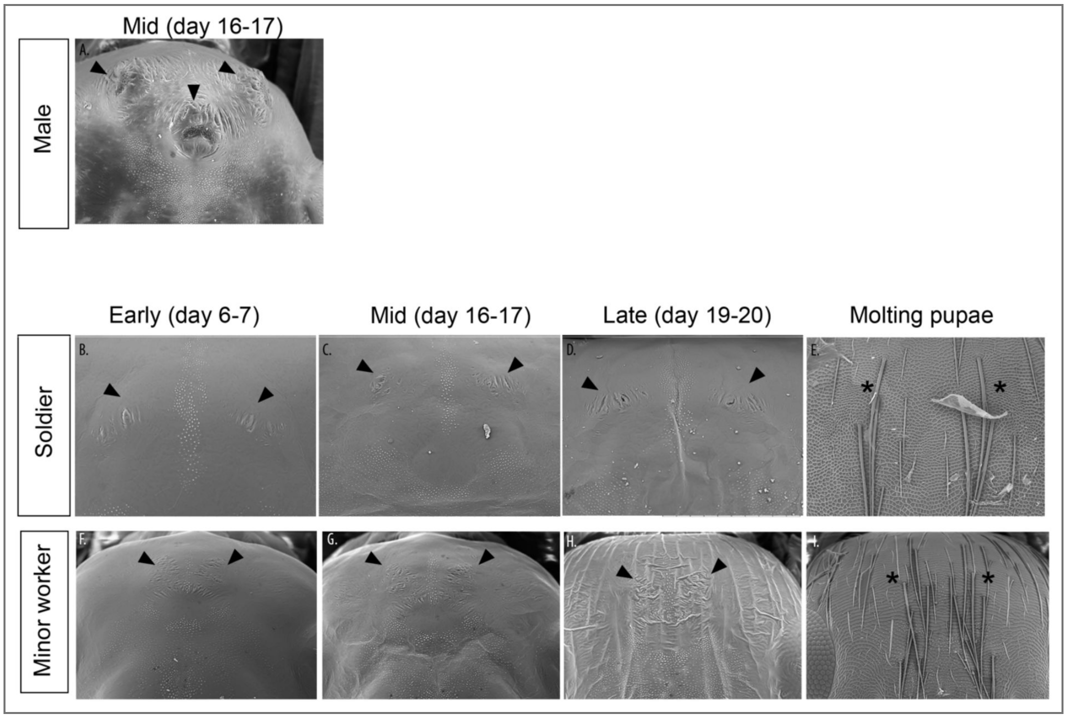


Figure 7. **Development of rudimentary ocelli in worker and soldier pupae of *C. floridanus*.**

(A) SEM showing ocelli development in males at mid-stages of pupal development. SEM showing development of rudimentary ocelli on (B to E) soldiers and (F to I) minor workers during early (day 6-7), mid (day 16-17), and late (19-20) pupal development. These ocelli rudiments disappear prior to adult stage (E and I).

expression of this GRN in developing workers lacking ocelli, this cannot solely explain how this latent expression became actualized (released and fixed) and what maintained it over millions of years.

One hypothesis for how this ocelli GRN became latently expressed and has been maintained in workers lacking ocelli is pleiotropy, which potentially results from the multiple roles that genes within the ocelli GRN play within the same imaginal disc (the eye-antennal disc). For instance, *otd-1* and *hh* also determine the regional identity of the head capsule, while *hh*, *toy*, *eya* and *so*, also play key roles in compound eye development in specifying structures such as optic lobes, cone differentiation, and rhabdomeres development (Blanco et al., 2009; Domínguez-Cejudo & Casares, 2015; Jean-Guillaume & Kumar, 2022). Another example is the highly conserved developmental regulatory gene *sonic hedgehog* (*shh*), which plays a key role in limb development across animals. The vestigialization of hindlimbs in snakes and therefore the re-evolution of hindlimbs in extinct species across the phylogeny of the group, is thought to be maintained during development through pleiotropic enhancers that drive *shh* expression. This means that the same enhancer (ZRS) that drives *shh* expression in the external genital, also drives it (pleiotropically) in the developing limb region of snakes (Leal & Cohn, 2018). Future studies should attempt to explore whether expression of these conserved genes in multiple regions of the eye-antennal disc is driven by shared enhancers. Alternatively, we cannot rule out the hypothesis that ocelli GRN has been co-opted to play a novel, yet currently unknown, function during worker larval development. Recent discoveries on the evolution of the wing GRN in ants provides support for this hypothesis. The wings, another nearly universal caste-specific trait in ants, develop in the reproductive male and queen caste, but are halted in the worker caste in response to environmental cues (Abouheif & Wray, 2002). The wing GRN is also found to be latently expressed in the wingless worker caste of ants and was thought to be functionless (Abouheif & Wray, 2002). However, it was recently discovered that the latent expression of this wing GRN in wingless worker caste acquired a novel function to generate big-headed soldiers in the hyperdiverse ant genus *Pheidole* (R. Rajakumar et al., 2012, 2018).

Finally, our inference that this latent expression of the ocelli GRN in workers facilitated the partial reversion to a single medial ocellus is supported by: (1) the close phylogenetic relationship between species that lack ocelli in adult workers but retain a latent expression of the ocelli GRN (*C. floridanus* and *P. rastellata*), and those that underwent a partial phylogenetic reversion to a single medial ocellus (*C. gibbinotus*, *D. gigas* and *P. bihamata*); (2) the presence of a developmental capacity or potential of the latent ocelli GRN expression to produce rudimentary ocelli in the pupal stage of *C. floridanus* workers that completely lack ocelli as adults; (3) the ability to experimentally induce only 1, only 2, or all 3 ocelli in similarly sized adult workers that normally lack them by applying high doses of Juvenile Hormone (JH) to worker-destined larvae in the ant *Monomorium pharaonis* (Li et al., 2024); and finally (4) in nature, the rare induction of a single medial ocellus by mermithid parasites in soldiers of *Pheidole pallidula* that typically lack ocelli in natural colonies (Laciny et al., 2019; Passera, 1976). The natural or experimental induction of worker individuals with only a single medial ocellus in different ant species also supports the inference that the single medial ocellus can be developmentally dissociated from the other two lateral ocelli. This suggests that reversion can facilitate the appearance of novel patterns of ocelli development in the workers if selected for.

In *C. floridanus* and *P. rastellata*, there is a latent ocelli GRN expression for all 3 ocelli, providing a springboard to facilitate the partial phylogenetic reversion to a single medial ocellus in *C. gibbinotus*, *P. bihamata* and *D. gigas*. In the genus *Polyrhachis*, however, some species in the same subgenus as *P. bihamata*, such as *P. bellicosa*, have three ocelli. Because the phylogenetic relationships within this subgenus have yet to be resolved, the independent reversion of ocelli in the ancestor of this subgenus may have resulted either in a single ocellus as reflected in *P. bihamata* or in 3 ocelli as reflected in *P. bellicosa* and 2 ocelli were subsequently lost giving rise to the single medial ocellus in *P. bihamata* (Hung, 1967). These possibilities further reinforce the different evolutionary pathways by which this latent potential may facilitate novelty after reversion.

Future functional, genomic, and comparative analyses of the ocelli GRN between larval stages, individuals within the worker caste, and species will ultimately reveal the architecture of the ocelli GRN and whether its underlying enhancers and promoters are modular or pleiotropic. This, in combination with manipulations of insect hormones, such as JH and ecdysone, would also elucidate whether variation in the size, presence/absence, number of ocelli is regulated by continuous or switch-like developmental mechanisms. And finally, determination the organismal and ecological function of the ocelli will be important to understand the adaptive significance of the latent expression of the ocelli GRN at both the individual and colony-level.

More broadly, our findings suggest that the ancestral and latent GRN expression (also known as ancestral developmental potential) we observed may generally underlie polyphenic systems, including caste-specific traits in ants and other eusocial organisms. We therefore propose that ancestral developmental potentials facilitate the re-evolution of polyphenic traits (West-Eberhard, 2003), and when these potentials facilitate only the partial re-evolution of alternative phenotypes, novel phenotypic patterns appear. We hope our findings not only inspire future work testing these proposals in polyphenic organisms, but also in non-polyphenic ones, where the polyphenism occurs at the cellular level but not at the level of the whole organism. Here, the cellular polyphenism produces alternative cell-types from a single genome in response to internal cues, such as morphogen gradients within the organism. If alternative cell types retain homologs or serial homologs of specific trait (Jackman et al., 2025; V. J. Lynch, 2023), then this raises the possibility that ancestral and latent developmental potentials may generally facilitate re-evolution of alternative cell types in multicellular organisms.

Methods

Ant maintenance and collection

Colonies of *C. floridanus collected at Gainesville* (*Florida*, USA) and *P. rastellata* collected at Mae Tang (Chiang Mai, Thailand), were maintained in plastic boxes with glass test tubes filled with water-constrained cotton wool. They were fed mealworms and the Bhatkar–Whitcomb diet (Bhatkar & Whitcomb, 1970). Colonies were maintained at 25°C with 60% humidity in complete darkness.

Larvae fixation and *in-situ* HCR

Gene sequences were obtained from NCBI GenBank database(Sayers et al., 2022) using genome BLAST against the assembled *C. floridanus* genome: *eyeless (ey*; XM_025414466), *distal-less (dll*; XM_025412727.1), *hedgehog, (hh*; XM_011262474.3), *eye absent (eya*; XM_025414466), *sine oculis (so;* XM_011252868.3) and *twin of eyeless (toy*; XM_011268499.3) genes. For *otd*, two paralogs of the gene were found in ants (XM_020028684.2 and XM_025415314.1), which is a result of a gene duplication event that has also been reported in wasps, bees, and beetles (J. A. Lynch et al., 2006; The Honeybee Genome Sequencing Consortium, 2006; Zattara et al., 2017). The two *otd* paralogs sequences were then aligned by multiple sequence alignment using all of the known *orthodenticle* related sequences in insects; *Drosophila melanogaster* (NM_001369965.1), *Apis mellifera*: *otd-1* (XM_026446161.1), *otd-2*(XM_006571236.3), *Nasonia vitripennis*: *otd-1* (XM_008212114.4), *otd-2* (XM_031926951.2) and *Tribolium castaneum otd-1* (XM_008192467.2), *otd-2* (XM_008192470.2) and *Acythosiphon pisum* (XP_008180802.1). To determine the *otd-1* paralog to *Drosophila melanogaster* (*otd-1)* a maximum likelihood gene tree was inferred using genetic distance model Hasegawa Kishino Yano (HKY) and 500 bootstrap replicates as incorporated in MEGA12 alpha (Kumar et al., 2024)(Figure S1). Probes corresponding to all genes were chosen for the hybridization chain reaction experiments using the fluorescence Hairpins (B1 546, B2 488, B3 647) synthesized by Molecular Instruments.

First, second, third and fourth larval instars of soldier, and minor worker-destined larvae and fourth instar of male destined larvae of *C. floridanus* and worker larvae of *P. rastellata* were collected and subsequently fixed in a PEM 4% formaldehyde solution for 2 hrs at room temperature. Fixed samples were then dehydrated progressively in methanol baths (25%, 50%, 75% methanol for 15 min each, and 100% overnight at 4°C) and stored in 100% methanol at −30°C until use. All gene expression analyses were conducted by In situ Hybridization Chain Reaction (HCR), following the protocol for HCR (v3.0 protocol) (Schwarzkopf et al., 2021). After the tissue was pre-hybridized in a prewarmed Probe Hybridization Buffer (Molecular Instruments) for 30 minutes at 37°C and incubated with HCR probes in a Probe Hybridization Buffer overnight at 37°C. Tissues were washed the next day in a prewarmed Probe Wash Buffer four times, 15 minutes each and washed in 5X SSCT (UltraPure 20XSSC Buffer, Invitrogen, diluted in water) three times for 5 minutes at room temperature. Tissues were pre-amplified in Amplification Buffer (Molecular Instruments) for 30 minutes at room temperature and incubated with snap-cooled HCR hairpins in Amplification Buffer overnight at room temperature. Tissues were then washed with 5X SSCT at room temperature twice for 5 minutes, for 30 minutes, and once for 5 minutes before being mounted on glycerol-DAPI 80%.

Microscopy

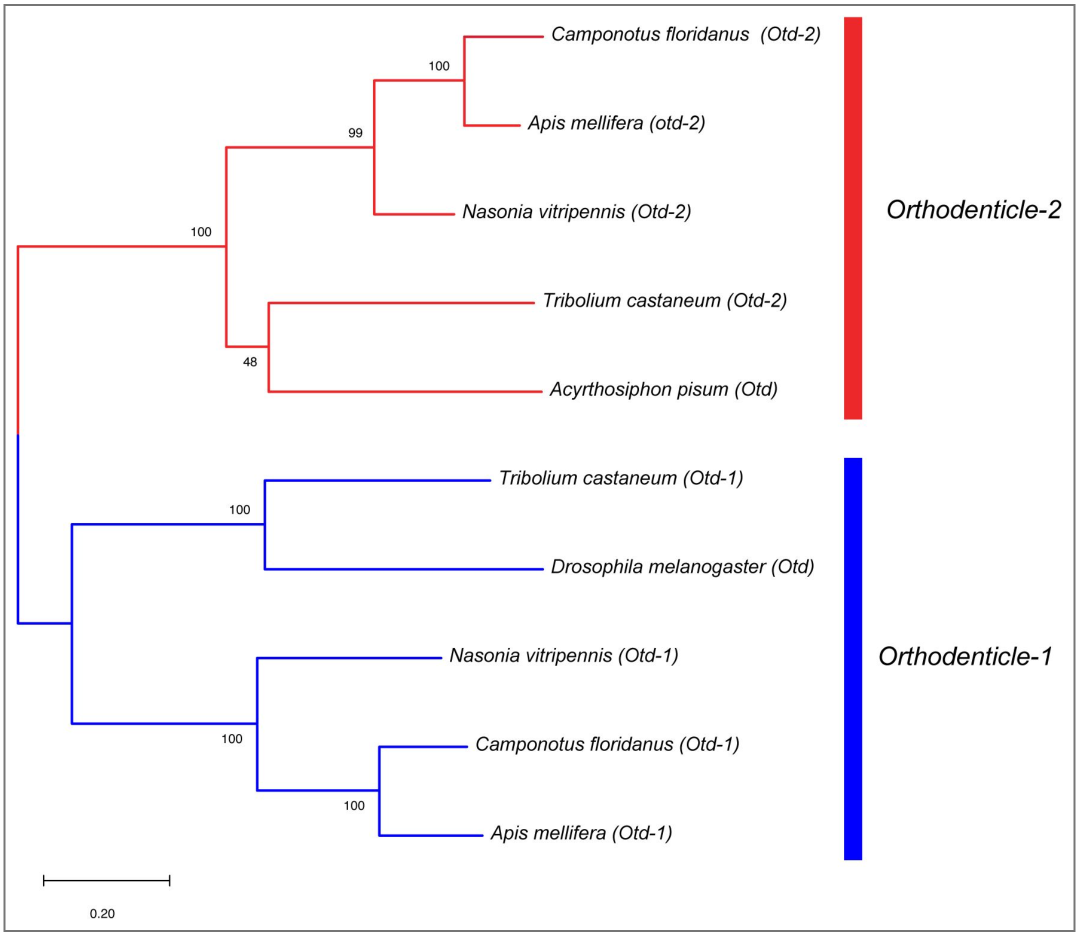
Confocal imaging was used to describe gene expression using Leica SP8 confocal microscope. Fiji (Schindelin et al., 2012) was used for image processing. Scanning electron microscopy (SEM) was done on a Hitachi TM3030 Scanning Electron Microscope.

Evolution of Ocelli in the Formicine Clade

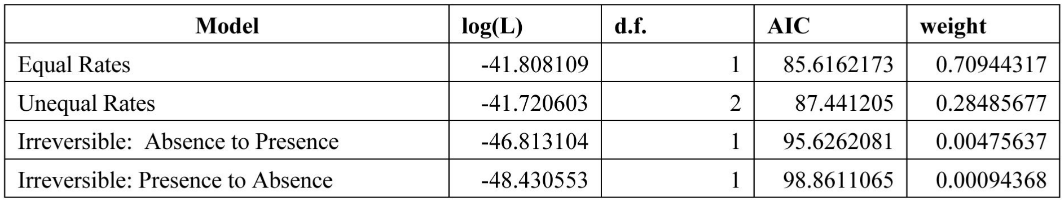
The evolution of ocelli on workers across the subfamily Formicinae was inferred using ancestral reconstruction (ASE) for discrete traits incorporated in the R package Phytools 4.3.3(Revell, 2024). The ASE analysis was based on the UCE70 phylogeny for the cade Formicinae published by Blaimer *et.al.* (Blaimer et al., 2015). The species *Camponotus floridanus* and *Polyrachis bihamata* were added manually to the phylogeny. To determine the presence or absence of ocelli in workers, photographs of the studied species from the database AntWeb,Version 8.114 were used (AntWeb, 2026). The observations from the database were contrasted with published information from the literature (Table S2). Ocelli were classified as present in the worker caste if individuals exhibit any of the 3 ocellus (2 lateral and 1 medial ocellus). In the case of the presence of worker polymorphism, ocelli were classified as present if any one of the 3 ocelli was present within any of the worker subcastes. Whereas the absence was the complete lack of ocelli across workers and soldiers.

Four separate models of ocelli evolution were tested for each character in phytools: Equal rate “ER”, all transitions rate different “ARD”, and an irreversible model allowing only transitions between presence and absence, and another irreversible model allowing only transitions between absence and presence. We compared the fit of our models by computing Akaike information criterion (AIC) and Akaike weights and conducting pairwise likelihood ratio tests. The new function incorporated in phytools 4.3.3, *simmap,* was used to generate stochastic character maps under each of the four models tested (see Table S1). The stochastic mapping that resulted from the stochastic simulation represented the frequencies that are equal to the weight of evidence supported by each model (Revell, 2024)

Supplementary figure and tables



Supplementary Figure 1. Simplified Gene tree based on maximum likelihood showing relationships between *orthodenticle (otd)* orthologs in *Drosophila melanogaster*, *Apis mellifera*, *Nasonia vitripennis*, *Tribolium castaneum, Acythosiphon pisum and Camponotus floridanus*. Branch values are bootstrap support (%). Colors: *otd-2* (red), *otd-1* (blue)



Model	log(L)	d.f.	AIC	weight
Equal Rates	-41.808109	1	85.6162173	0.70944317
Unequal Rates	-41.720603	2	87.441205	0.28485677
Irreversible: Absence to Presence	-46.813104	1	95.6262081	0.00475637
Irreversible: Presence to Absence	-48.430553	1	98.8611065	0.00094368

Supplementary Table 1. Model selection under maximum likelihood estimation implemented in phytools (Revell, 2024).

The results are ordered by decreasing Akaike Weights (w).

Subfamily	**Genus**	**Species**	**Ocelli Count**	**References**
outgroup	*Acanthoponera*	*minor*	0	AntWeb.org
Formicinae	*Acropyga*	*acutiventris*	0	AntWeb.org
outgroup	*Aneuretus*	*simoni*	0	AntWeb.org
Formicinae	*Anoplolepis*	*custodiens*	0	AntWeb.org
Formicinae	*Anoplolepis*	*gracilipes*	0	AntWeb.org
Formicinae	*Aphomomyrmex*	*afer*	3	AntWeb.org
Formicinae	*Bajcaridris*	*theryi*	3	Santschi, 1936
outgroup	*Brachymyrmex*	*depilis*	0	AntWeb.org
Formicinae	*Calomyrmex*	*albertisi*	0	AntWeb.org
Formicinae	*Calomyrmex*	*laevissimus*	0	AntWeb.org
Formicinae	*Camponotus*	*floridanus*	0	AntWeb.com
Formicinae	*Camponotus*	*gibbinotus*	1	AntWeb.org
Formicinae	*Camponotus*	*hyatti*	0	MacKay & Mackay, 2002
Formicinae	*Camponotus*	*maritimus*	0	Ward, 2005
Formicinae	*Colobopsis*	*saundersi*	0	AntWeb.org
Formicinae	*Colobopsis*	*vitiensis*	0	Mann, 1920
Formicinae	*Cataglyphis*	*cursor*	3	AntWeb.org
outgroup	*Cladomyrma*	*petalae*	0	Agosti, 1991
outgroup	*Dolichoderus*	*pustulatus*	0	AntWeb.org
Formicinae	*Dinomyrmex*	*gigas*	1	AntWeb.org
Formicinae	*Echinopla*	*australis*	0	AntWeb.org
Formicinae	*Euprenolepis*	*procera*	0	Lapolla, 2009
Formicinae	*Formica*	*moki*	3	Cole Jr, 1943
Formicinae	*Formica*	*neogagates*	3	AntWeb.org
Formicinae	*Gigantiops*	*destructor*	3	Smith, 1858
Formicinae	*Iberoformica*	*subrufa*	3	Antwiki- genus
Formicinae	*Lasiophanes*	*atriventris*	3	AntWeb.org
Formicinae	*Lasius*	*californicus*	0	AntWeb.org
Formicinae	*Lasius*	*niger*	3	AntWeb.org
Formicinae	*Lepisiota*	*canescens*	3	Sharaf et al., 2020
outgroup	*Manica*	*bradleyi*	0	AntWeb.org
outgroup	*Myrmecia*	*pyriformis*	3	AntWeb.org
Formicinae	*Myrmecocystus*	*flaviceps*	3	AntWeb.org

Supplementary Table 2. Database with the references used in this study for presence and number of ocellus (1) and absence (0) of ocellus.

Formicinae	*Myrmecorhynchus*	*emeryi*	3 *(soldiers and media, absent in minors)*	Wheeler, 1917
outgroup	*Myrmelachista*	*flavocotea*	0	AntWeb.org
Formicinae	*Myrmoteras*	*iriodum*	3	AntWeb.org
outgroup	*Nothomyrmecia*	*macrops*	0	AntWeb.org
Formicinae	*Notoncus*	*capitatus*	3	AntWeb.org
Formicinae	*Notostigma*	*carazzii*	3	AntWeb.org; Emery, 1920
Formicinae	*Nylanderia*	*dodo*	0	Lapolla et al., 2011
Formicinae	*Nylanderia*	*hystrix*	3	Kallal & LaPolla, 2012
Formicinae	*Oecophylla*	*longinoda*	0	AntWeb.org
Formicinae	*Oecophylla*	*smaragdina*	0	Cole & Jones, 1948
Formicinae	*Opisthopsis*	*respiciens*	0	AntWeb.org
Formicinae	*Paraparatrechina*	*glabra*	3	AntWeb.org
Formicinae	*Paraparatrechina*	*oceanica*	0	AntWeb.org
Formicinae	*Paratrechina*	*antsingy*	3	LaPolla & Fisher, 2014
Formicinae	*Paratrechina*	*longicornis*	3	AntWeb.org
Formicinae	*Paratrechina*	*zanjensis*	3	LaPolla et al., 2013
Formicinae	*Petalomyrmex*	*phylax*	3	Snelling, 1979
Formicinae	*Plagiolepis*	*alluaudi*	0	AntWeb.org
Formicinae	*Polyergus*	*breviceps*	3	AntWeb.org; Smith, 1947
Formicinae	*Polyrhachis*	*decumbens*	0	Kohout, 2006
Formicinae	*Prenolepis*	*emmae*	3	AntWeb.org
Formicinae	*Prenolepis*	*imparis*	0	Williams & LaPolla, 2016
Formicinae	*Proformica*	*mongolica*	3	AntWeb.org
Formicinae	*Prolasius*	*convexus*	3	McAreavey, 1947
Formicinae	*Pseudolasius*	*australis*	0	Emery, 1925
Formicinae	*Pseudonotoncus*	*hirsutus*	3	Shattuck & O'Reilly, 2013
outgroup	*Rhytidoponera*	*chalybaea*	0	AntWeb.org
Formicinae	*Rossomyrmex*	*anatolicus*	3	AntWeb.org
Formicinae	*Santschiella*	*kohli*	3	Forel, 1916
Formicinae	*Teratomyrmex*	*greavesi*	3	Shattuck & O'Reilly, 2013
outgroup	*Tetraponera*	*rufonigra*	3	Ward, 2001
Formicinae	*Zatania*	*albimaculata*	3	AntWeb.org

Supplementary Table 2. (continued)

Data availability

All data reported in this paper are provided in Table S2 in the supplemental information. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the corresponding Author upon request.

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Additional information

Author Contribution

AV-C and EA conceived the project. AV-C and JA gathered ocelli data. AV-C and JA conducted ancestral state reconstruction. A V-C conducted HCR. TC performed SEM imaging. AV-C and EA wrote the manuscript.

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Peer reviews

Reviewer #2 (Public review):

Summary:

The manuscript titled "Latent gene network expression underlies partial re-evolution of a polyphenic trait in the worker caste of ants" by Vasquez-Correa et al. aimed to study genetic mechanisms underlying developmental plasticity, especially binary polyphenism in queen vs worker ant castes. This is an interesting question regarding the extent to which phenotypic traits were altered, lost or regained, and how molecular pathways (upstream vs. downstream) can facilitate this process.

In ants, reproductive castes (queens and males) develop wings as well as 3 ocelli for mating flights and other activities, while worker castes are wingless, and in some species, they have either no or a reduced number of ocelli. The phylogenetic analysis showed that in the Camponotini ant clade, the one-ocellus phenotype re-evolved in three species independently. The authors analyzed the conserved developmental pathways between Drosophila (well-established) and ants using HCR (a high-quality in situ hybridization technique). They found that although upstream genes for the development of ocelli (otd and hh) showed similar expression between castes, downstream genes (toy, eya, and so) had reduced or no expression in workers of C. floridanus, and this differential expression may lead to partial or complete loss of ocelli. Consistently, workers develop rudimentary tissues, suggesting that they initiate the ocellus developmental process but somehow stop it before adulthood.

Strengths:

Evo-devo approaches to reveal conserved molecular pathways of ocellus development. High-quality HCR provided convincing evidence of the expression of key genes in ocelli, eyes and antenna throughout larval development.

Using HCR, the authors showed differential expression of downstream genes in males vs. soldiers vs. minor workers of C. floridanus, which might explain phenotypic differences between castes.

Comments on revised version.

The authors have addressed the concerns in the revision. No further comments.

https://doi.org/10.7554/eLife.110148.2.sa2

Reviewer #3 (Public review):

Summary:

This paper examines the loss and re-evolution of specific organs during the evolution of ants. The authors show that these organs, the ocelli, disappear and are re-evolved in different ant species, and in different ant castes within these species. The Authors show that this is linked to a conserved GRN discovered in Drosophila, that appears to underlie the development of the ocelli, and demonstrate that this GRN appears to remain active in the developing heads of ants that have no ocelli- implying that it is the evolutionary latency of this GRN that allows loss and subsequent evolution.

Strengths:

This manuscript has outstanding imaging of a very difficult developing organ, and the key data, fluorescence in situ hybridisation, is done well and clearly shows what the authors wish to demonstrate. The methods are well described and underpin the whole work.

The authors convincing demonstrate that gene expression patterns imply the conservation of the ocellus gene regulatory network from Drosophila to ants. They further show that this network is present even in ants that don't produce an adult ocellus, but do show that in those species, loss of a developing nascent ocellus (which they identify) occurs at the same time as an interruption in the expression of the key genes in the GRN. All of this data is beautifully presented and explained.

Weaknesses:

There is one key weakness in that there are no functional students that indicate that the GRN actually does make the ocellus, though the expression patterns are convincing. This applies to loss of the ocellus as well. It would be nice to see that transient loss of the ocelli GRN might lead to loss of ocelli in ant species that have them. These are very difficult things to achieve as the key genes have earlier developmental roles, such that CRISPr knockouts would not be interpretable, and transient RNAi in the head capsules of developing pupal ants would be challenging.

As the authors note in their response this is very difficult to achieve. While the addition of this data would raise this manuscript to an outstanding one, I think the data presented is solid, well-presented and provides novel insight.

https://doi.org/10.7554/eLife.110148.2.sa1

Author response:

The following is the authors’ response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The manuscript by Vasquez-Correa and colleagues describes the expression pattern of the ocelli (simple eye) gene regulatory network in ants. They correlate the expression pattern of these genes with the presence and absence of ocelli in different classes and species of ants. The presence of ocelli is a polyphenic trait in ants - understanding the*

> *molecular and developmental underpinnings of polyphenic traits is of significant interest to evolutionary biologists, developmental biologists, and ecologists. The authors propose that the presence of the latent expression of the ocellar network in classes of ants that do not display ocelli in the adults may underlie the re-evolution of ocelli within the ant lineage.*
>
> *Strengths:*
>
> *The strengths of the manuscript are that it is well written, the images are of the highest quality, and the data support the conclusions of the authors.*

We thank Reviewer 1 for their positive comments.

> *Weaknesses:*
>
> *One improvement that could be made is to include imaginal discs of the queen ants as well as scanning electron images of the ocelli of the queen ant to match the pupal stage images of the worker and soldier ants. A second improvement is to attempt a gene knockdown using RNAi or similar methods to ensure that the genes that are being studied are, in fact, responsible for ocelli development in the ant.*

The reproductive caste in ants is typically composed of both winged males and winged queens. We agree with Reviewer 1 that the queen caste, which develop 3 fully functional ocelli, is an important point of comparison in our study to the wingless minor workers and soldiers. Unfortunately, however, laboratory colonies rarely produce reproductive queens, and in the field, queen production in colonies of *C. floridanus* occurs within a narrow seasonal window, making the collection of queen larvae particularly challenging for developmental work. In contrast, the winged males, which also develop 3 functional ocelli like the queens for help during mating flights, can be readily generated in the lab throughout the year. Therefore, we use males as a proxy for characterizing ocelli development and GRN in queens and the winged reproductive caste as a whole. Given the deeply conserved gene regulatory networks underlying this trait across insects, we believe this is a reasonable assumption.

We also agree with Reviewer 1 that using RNAi to knock down genes in the ocelli GRN would improve the study. For completeness of the scientific record, we would like reviewers and readers to know that we actually did, in fact, invest significant effort trying to knock down *otd-1* (ortholog of the *Drosophila otd* gene), which functions as key upstream regulator of ocellar development. In *Drosophila*, RNAi knockdown of *otd* disrupts the development of all three ocelli as well as fine morphological features on the anterior of the head. In *C. floridanus*, *otd -1* is expressed in the head capsule and brain (see Author response image 1 in this response). Injection of dsRNA of *otd*-1into whole soldier-destined larvae, significantly reduced *otd -1* expression in the brain relative to its control, while in the head capsule, *otd -1* expression remained largely unchanged relative to its control (see Author response image 1 in this response). This indicates that in the same individual, the injected *otd -1* dsRNA was able to penetrate and significantly reduce *otd -1* expression in the brain, but, was unable to penetrate the head capsule, where *otd -1* expression remained largely unchanged. No ocellar phenotypes could be observed in pupae or adults. Therefore, for technical (not biological) reasons, we were unable to knockdown genes in the ocelli GRN in the head capsule. We hope to solve this technical problem in the coming years to add a mechanistic explanation for the latent expression and maintenance of the ocelli GRN in workers that completely lack ocelli as adults.

> ***Reviewer #2 (Public review):***
>
> *Summary:*

> *The manuscript titled "Latent gene network expression underlies partial re-evolution of a polyphenic trait in the worker caste of ants" by Vasquez-Correa et al. aimed to study genetic mechanisms underlying developmental plasticity, especially binary polyphenism in queen vs worker ant castes. This is an interesting question regarding the extent to which phenotypic traits were altered, lost or regained, and how molecular pathways (upstream vs. downstream) can facilitate this process.*
>
> *In ants, reproductive castes (queens and males) develop wings as well as 3 ocelli for mating flights and other activities, while worker castes are wingless, and in some species, they have either no or a reduced number of ocelli. The phylogenetic analysis showed that in the Camponotini ant clade, the one-ocellus phenotype revolved in three species independently. The authors analyzed the conserved developmental pathways between Drosophila (well-established) and ants using HCR (a high-quality in situ hybridization technique). They found that although upstream genes for the development of ocelli (otd and hh) showed similar expression between castes, downstream genes (toy, eya, and so) had reduced or no expression in workers of C. floridanus, and this differential expression may lead to partial or complete loss of ocelli. Consistently, workers develop rudimentary tissues, suggesting that they initiate the ocellus developmental process but somehow stop it before adulthood.*
>
> *Strengths:*
>
> *Evo-devo approaches to reveal conserved molecular pathways of ocellus development. High-quality HCR provided convincing evidence of the expression of key genes in ocelli, eyes and antenna throughout larval development.*
>
> *Using HCR, the authors showed differential expression of downstream genes in males vs. soldiers vs. minor workers of C. floridanus, which might explain phenotypic differences between castes.*

We thank Reviewer 2 for their positive comments.

> *Weaknesses:*
>
> *Although the molecular pathway is conserved, the mechanism underlying the lack of ocelli in workers remains unclear. In C. floridanus, it could be explained by the evidence of no expression of certain developmental genes, but in other species, e.g. Polyrachis rastellata, is their expression intact, or reduced? There is no control male.*
>
> *In addition, HCR in species with partial re-evolution (if their genomes have been sequenced) would be useful to understand the mechanism. For example, there might be differential spatial expression between medial and lateral ocelli.*

We agree with Reviewer 3 that investigating the mechanisms underlying the lack of specific ocelli in these and other species is the next step for this research. Here, our main focus was instead on trying to explain the mechanisms underlying partial reversion of ocelli through the persistence of ocelli GRN expression in adult workers lacking ocelli. We therefore focused on the latent expression of the ocelli GRN in *Polyrachis rastellata*, a species that completely lack ocelli in adult workers, and how it may have facilitated the partial reversion of a single ocellus in its congener *Polyrachis bihamata*. Therefore, although we did not reveal specific interruption points in the ocelli GRN in *Polyrachis rastellata*, our results showing that this species expresses three genes of the ocelli GRN, offers sufficient evidence that this network is conserved and likely facilitated the partial reversion to a single ocellus in *P. bihamata*.

We also agree with Reviewer 3 regarding the male control in *P. rastellata* and obtaining the species in our study that have undergone partial re-evolution. Unfortunately, these ants occur in Southeast Asia and are very difficult to collect. For males in *P. rastellata*, our colony died before we could try to induce male development. However, given the deep conservation of the network in the males of a genus within the same subfamily (Camponotini), we feel it is reasonable to assume that the network would also be conserved in the males of *P. rastellata*, especially since the genes we sampled are conserved in workers that do not develop ocelli as adults. As am sure the Reviewer may know that this is a continual challenge of working with emerging models in evodevo.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *This paper examines the loss and re-evolution of specific organs during the evolution of ants. The authors show that these organs, the ocelli, disappear and are re-evolved in different ant species and in different ant castes within these species. The authors show that this is linked to to a conserved GRN discovered in Drosophila, that appears to underlie the development of the ocelli, and demonstrate that this GRN appears to remain active in the developing heads of ants that have no ocelli- implying that it is the evolutionary latency of this GRN that allows loss and subsequent evolution.*
>
> *Strengths:*
>
> *This manuscript has outstanding imaging of a very difficult developing organ, and the key data, fluorescence in situ hybridisation, is done well and clearly shows what the authors wish to demonstrate. The methods are well described and underpin the whole work.*
>
> *The authors convincing demonstatrate that gene expression patterns imply the conservation of the ocellus gene regulatory network from Drosophila to ants. They further show that this network is present even in ants that don't produce an adult ocellus, but do show that in those species, loss of a developing nascent ocellus (which they identify) occurs at the same time as an interruption in the expression of the key genes in the GRN. All of this data is beautifully presented and explained.*

We thank Reviewer 3 for their positive comments.

> *Weaknesses:*
>
> *There is one key weakness in that there are no functional students that indicate that the GRN actually does make the ocellus, though the expression patterns are convincing. This applies to loss of the ocellus as well. It would be nice to see that transient loss of the ocelli GRN might lead to loss of ocelli in ant species that have them. These are very difficult things to achieve, as the key genes have earlier developmental roles, such that CRISPR knockouts would not be interpretable, and transient RNAi in the head capsules of developing pupal ants would be challenging.*

We agree with Reviewer 3 that functional experiments in species where workers both have ocelli present and absent is a key next step in this research. Please see our response to Reviewer 1 on our failed attempts to achieve this. We are therefore grateful to Reviewer 3 for acknowledging the challenges in trying to establish RNAi and CRISPR in the head capsules of developing workers in these ants. Also, please see our response to Reviewer 2 on the difficulty of finding and collecting these ants, which occur mainly in Southeast Asia.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***

> *One improvement that could be made is to include imaginal discs of the queen ants as well as scanning electron images of the ocelli of the queen ant to match the pupal stage images of the worker and soldier ants.*
>
> *A second improvement is to attempt a gene knockdown using RNAi or similar methods to ensure that the genes that are being studied are in fact responsible for ocelli development in the ant.*

Please see our response to Reviewer 1 above.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *For the questions below, if there is no experimental evidence, consider addressing them in the Discussion.*
>
> *Do sizes of ocelli different between castes? For example, even workers have 1-3 ocelli, their sizes are smaller than those of males/queens, especially in workers with 1 ocellus. If so, might it be continuous (not binary) changes in downstream gene expression that control ocellus size, with no ocellus below threshold? Does this favor the hypothesis of threshold but not switch?*

We thank Reviewer 3 for highlighting an important point about the size and development of ocelli. Observations suggest that ocelli tend to be larger in queens and males than in workers and soldiers in species with ocelli. However, we lack quantitative data to test this conclusively. We now include a sentence on the Discussion stating that an important avenue of future work should investigate whether threshold or switch mechanisms influencing the presence/absence, as well as size, of ocelli between queens and workers.

> *For the species whose workers have a single ocellus, are there variations, e.g. spanning from 0, 1 to 2? If 2, always one medial plus one of the two laterals? If always one, it would be a good control for staining to see up- vs down-regulation of downstream gene expression within the same individual.*

We agree with Reviewer 2 that this is a fascinating approach to our question. We have not observed natural wild-type variation in the number of developing ocelli in the same-sized individuals in the worker caste. However, in a distantly related leaf-cutting ant species (*Atta cephalotes*) belonging to different subfamily (the Myrmicinae) individuals with different head-to-body scaling within the same colony can vary in the number of ocelli. For example, soldiers of *Atta cephalotes* include individuals developing one, two, or three ocelli. These configurations can appear as only the median ocellus, only the two lateral ocelli, or even the median plus a single lateral ocellus. Interestingly, these correlations vary with changes in the size and head-to body scaling, suggesting that each ocellus can undergo different degrees of development, with one or more remaining vestigial or completely absent. On the other hand, workers in other species consistently develop a single ocellus, like in workers of *Polyrachis bihamata*, with no correlation to size or head-to-body scaling. These cases highlight how evolutionarily labile this trait is among workers of different ant species, which supports our proposal that the underlying gene regulatory network remains latent, thereby facilitating the emergence of novel trait combinations. We therefore agree on the importance of comparing the developmental mechanisms underlying these patterns temporally across larval stages and between individuals within a colony. We have now incorporated 2 sentences into the discussion, stating that this will be an important avenue for future work.

> *Is there any function of a single ocellus in workers, or just a consequence of incomplete down-regulation of gene expression?*

Thank you again for highlighting these important points that help us to elaborate on the discussion of our study. The functional role of ocelli in species that develop these structures remains largely understudied. However, for some species particularly within the Formicinae clade the function of the three ocelli in workers has been investigated, revealing that they serve as a celestial compass that facilitates navigation. We reference these findings in our Introduction and Discussion to illustrate that the presence of three ocelli in workers can represent an adaptive trait. In contrast, the functional significance of a single ocellus or of partially developed ocelli remains an important question. This knowledge gap presents a promising avenue for future research to understand the adaptive value of reduced, partially suppressed ocellar development. We have now added a sentence in the discussion stating this.

> *In previous studies, JH treatment can increase the number of ocelli in workers, consistent with its role in promoting reproductive development. In the ocellus developmental pathway, what causes the reduction of downstream gene expression in C. floridanus? Does JH directly regulate their expression?*

We thank Reviewer 2 for proposing yet another interesting question for future investigation, which we have added to the Discussion.

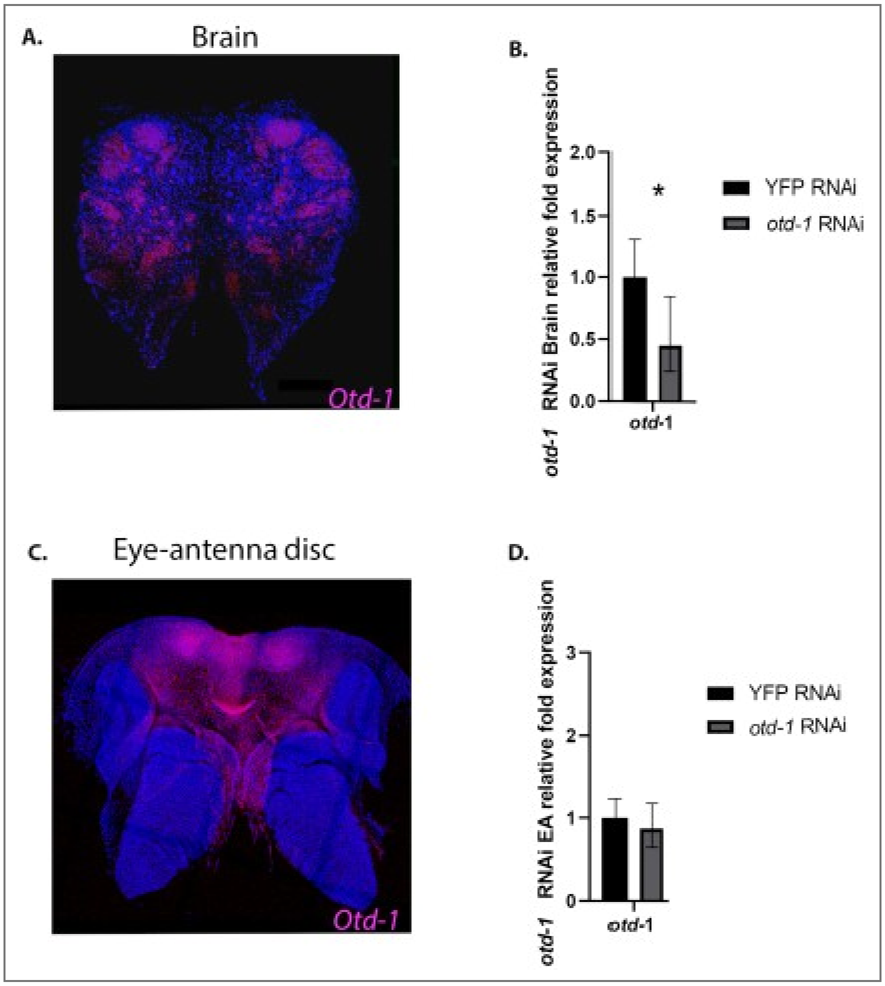
The only current evidence available in *C. floridanus* is a recent study (MacMillan et al. 2025), in which minor workers and soldiers were treated with JH at different developmental stages. Unfortunately, no evidence of ocelli induction was observed in JH-treated individuals, suggesting that the mechanisms of ocelli development in *C. floridanus* might be highly canalized, especially in species that exhibit worker polymorphism (inter-individual variation in size and head-to-body scaling within the worker cate). However, more studies are required to understand why in *Monomorium pharonis* (no worker polymorphism) ocelli development can be readily induced by JH, while in another *C. floridanus* (with worker polymorphism) it appears quite difficult.

> *"In D. melanogaster, the head develops from the eye-antenna disc" This statement is not correct. The brain does not belong to the eye-antennal disc.*

We thank Reviewer 2 for catching the misspelling. We have changed the name to eye-antenna disc in the sentence.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *It is hard to see the developing ocelli in Figure 7 - could the authors increase the contrast to make them more visible?*

We have made the suggested changes to Figure 7 in the main article, and it has indeed improved the figure.



Author response image 1. RNAi knockdowns in developing soldiers of *Camponotus floridanus* show a reduction of *otd -1* expression in the brain, but no effect on *otd -1* expression in the eye-antenna disc. A. HCR revealing *otd -1* expression in the brain B. qPCR of *otd -1* expression after RNAi knockdown shows significantly reduced *otd -1* expression in the brain, C. HCR revealing *otd -1* expression in the eye-antenna disc D. qPCR of *otd -1* expression after RNAi knockdown shows no significant affect on *otd -1* expression in the eye-antenna disc.

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