



# Regulatory Mechanisms Underlying the Differentiation of Neotenic Reproductives in Termites: Partial Release From Arrested Development

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### Specialty section:

This article was submitted to  
Behavioral and Evolutionary Ecology,  
a section of the journal  
Frontiers in Ecology and Evolution

**Received:** 30 November 2020

**Accepted:** 05 February 2021

**Published:** 25 February 2021

### Citation:

Oguchi K, Maekawa K and  
Miura T (2021) Regulatory  
Mechanisms Underlying  
the Differentiation of Neotenic  
Reproductives in Termites: Partial  
Release From Arrested Development.  
*Front. Ecol. Evol.* 9:635552.  
doi: 10.3389/fevo.2021.635552

Eusocial insects exhibit reproductive division of labor, in which only a part of colony members differentiates into reproductives. In termite colonies, the division of labors is performed among multiple types of individuals (i.e., castes), such as reproductives, workers, and soldiers to organize their society. Caste differentiation occurs according to extrinsic factors, such as social interactions, leading to developmental modifications during postembryonic development, and consequently, the caste ratio in a colony is appropriately coordinated. In particular, when the current reproductives die or become senescent, some immature individuals molt into supplementary reproductives, also known as “neotenic,” that take over the reproductive task in their natal colony. Neotenic exhibit variety of larval features, such as winglessness, and thus, immature individuals are suggested to differentiate by a partial release from arrested development, particularly in the reproductive organs. These neotenic features, which have long been assumed to develop via heterochronic regulation, provide us opportunities to understand the developmental mechanisms and evolutionary origin of the novel caste. This article overviews the accumulated data on the physiological and developmental mechanisms that regulate the neotenic differentiation in termites. Furthermore, the evolutionary trajectories leading to neotenic differentiation are discussed, namely the acquisition of a regulatory mechanism that enable the partial release from a developmentally arrested state.

**Keywords:** termite, caste differentiation, neotenic, modularity, heterochrony, developmental arrest

## INTRODUCTION: CASTE DIFFERENTIATION IN TERMITES

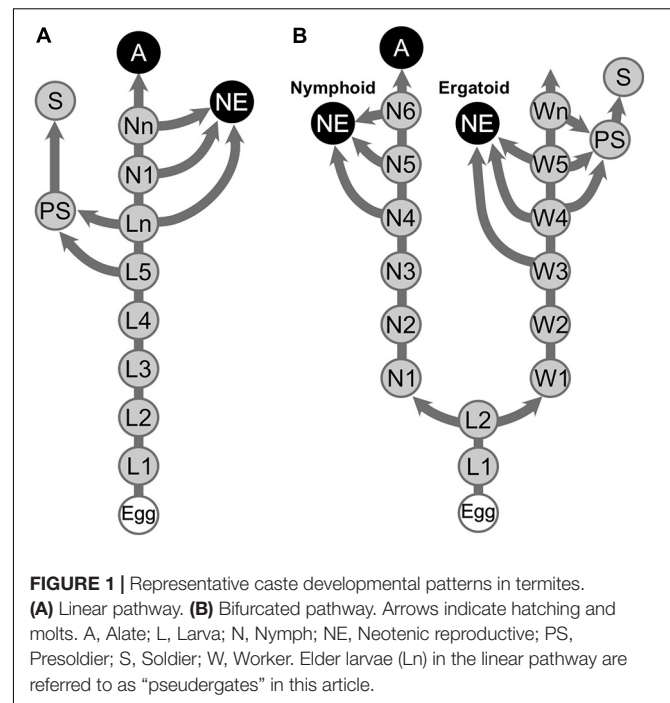
The acquisition of the caste system in social insects is a critical step in eusocial evolution, in which colony tasks are allocated to different types of individuals with caste-specific morphological features (Lin and Michener, 1972). Regulatory mechanisms underlying the development of caste-specific characteristics should be deeply associated with eusociality (Miura, 2005). In particular, the reproductive division of labor between the reproductive and sterile castes is one of the most distinctive features in eusocial insects (Wilson, 1971, 1975). Among eusocial insects, termites, which are distantly related to hymenopterans (Noirot, 1969; Korb, 2015), exhibit highly sophisticated and

complex societies. It is believed that the termite sociality first emerged in the Early Cretaceous, approximately 50 million years before the appearance of eusocial hymenopterans (Grimaldi and Engel, 2005; Roisin and Korb, 2011; Engel, 2015). Although regulatory mechanisms that control the differentiation of reproductive and sterile castes are thought to differ between hymenopterans and termites, most of the involved mechanisms are unclear, particularly in termites.

Generally, in hemimetabolous insects (e.g., cockroaches, locusts, etc.), adult-specific characteristics, such as compound eyes, wings, and reproductive organs, gradually develop during postembryonic development via moltings, and the dramatic morphological modifications occur at the final molt, i.e., imaginal molt (Anderson, 1972). In termites, caste-specific morphological characteristics are created through moltings (Noirot, 1969; Roisin, 2000), in which the developmental patterns differ depending on the caste fate (Miura, 2005; Korb and Hartfelder, 2008). Therefore, the regulatory mechanisms of molting and metamorphosis contribute to caste-specific development. Concerning the differentiation into alates, i.e., winged reproductives that form new colonies, imaginal characteristics, such as compound eyes, wings, and gonads, dramatically develop (Nutting, 1969; Weesner, 1969; Nii et al., 2019). Thus, the differentiation into alates is considered homologous to the imaginal development in other hemimetabolous insects (Nalepa and Bandi, 2000). In particular, termite colonies include numerous sexually immature individuals engaged in various tasks such as nursing and foraging (Thorne, 1996; Korb and Hartfelder, 2008). In these individuals, the development of imaginal organs like compound eyes, wings, and gonads is restricted or arrested during postembryonic development (Nalepa and Bandi, 2000; Bourguignon et al., 2016).



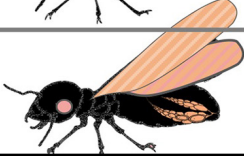
As caste differentiation fates are determined during postembryonic development in termites, the patterns of caste differentiation are often depicted as differentiation pathways. Among termite families, two major patterns of caste differentiation pathways are recognized, i.e., linear and bifurcated pathways (Figure 1; Noirot, 1969; Roisin, 2000; Bourguignon et al., 2014). Bifurcated pathways have been described in Mastotermitidae, Hodotermitidae, Termitidae, and some species of Rhinotermitidae, in which the differentiation point for alates and apterous individuals occurs at a relatively early stage of postembryonic development, i.e., first or second larval instars (Roisin, 2000). Workers in the bifurcated pathways cannot develop into alates, and thus, they are called “true workers,” although they still have the potential to develop into reproductives (Korb and Hartfelder, 2008; Bourguignon et al., 2016). In contrast, in the linear pathways observed in Archotermopsidae, Stolotermitidae, Kalotermitidae, and Serritermitidae, in addition to some species of Rhinotermitidae, elder larval individuals serve as “workers” while possessing the potential to differentiate into alates, and thus, they are called pseudergates, meaning “false workers” (Grassé and Noirot, 1947; Korb and Hartfelder, 2008).

In addition to these developmental stages, other derived pathways, i.e., soldier and neotenic differentiation pathways,



have also been investigated by many researchers. Both soldier and neotenic castes are novel developmental stages only present in termites, and these castes represent synapomorphies of the termite lineage, as they are present in all termite species with few exceptions (Noirot, 1969; Korb, 2015). Soldiers differentiate from workers or pseudergates through two molting events via a presoldier stage (Noirot, 1969; Roisin, 2000; Korb and Hartfelder, 2008). Because soldier differentiation can be artificially induced by the application of juvenile hormone (JH), several studies have described soldier developmental process in different species (Hrdý and Křeček, 1972; Howard and Haverty, 1979; Scharf et al., 2003). During soldier differentiation, the body plan of termites, particularly the anterior parts that are used for defensive behaviors, are largely modified through a specific morphogenesis (Miura and Matsumoto, 2000; Koshikawa et al., 2003; Watanabe and Maekawa, 2008; Toga et al., 2013). The morphogenetic process occurs downstream of hormonal and morphogenic factors, which are well documented (Cornette et al., 2008; Sugime et al., 2019; Miura and Maekawa, 2020). However, until recently, because methods for inducing neotenic differentiation had not been established, studies on neotenic differentiation are relatively sparse compared with those of soldier differentiation (Shimoji et al., 2017; Oguchi et al., 2020).

Neotenic reproductives are seen in nearly all the termite families, although they are occasionally lost in some genera (Myles, 1999; Bourguignon et al., 2016). Neotenic, also known as secondary reproductives, can also be referred to as replacement or supplementary reproductives, depending on situations of occurrence (Korb and Hartfelder, 2008). At the time when primary reproductives die or become senescent, they differentiate from immature individuals and

		Compound eyes	Wings	Genital structures	Gonads
<b>Pseudergate/Worker/Nymph</b>		—	—	—	—
<b>Neotenic reproductive</b>		—	—	+	+
<b>Alate</b>		+	+	+	+

**FIGURE 2** | Schematic illustrations of the developmental degree of imaginal organs in each caste. “+” indicates well-developed imaginal organs like alates. “—” indicates imaginal organs with suppressed or interrupted developments compared with those of alates.

exclusively engage in reproduction in their natal nests (Figure 1; Lüscher, 1961; Thorne, 1996; Korb and Hartfelder, 2008). Some species produce neotenic not only as replacements but also as backup against the loss of primary reproductives (Matsuura et al., 2009; Hellemans et al., 2019). Upon the differentiation of neotenic reproductives, adult-specific characteristics related to reproductive organs develop, whereas other imaginal features, such as wings, do not (Figure 2). Thus, it is suggested that the neotenic-specific morphology is accomplished by a heterochronic regulation, in which the development is accelerated or arrested depending on body modules (Nalepa and Bandi, 2000).

The differentiation of neotenic reproductives is controlled by genetic, maternal, and environmental factors. In *Reticulitermes speratus* and several other species, for instance, genetic, and maternal factors influenced by sex-linked genetic mechanisms, i.e., the asexual queen succession (AQS) system, are responsible for the neotenic differentiation (Hayashi et al., 2007; Matsuura et al., 2009; Matsuura et al., 2018; Hellemans et al., 2019), although environmental factors during the postembryonic development also affect the caste fate determination (Hayashi et al., 2007, 2013). In contrast, environmental factors during postembryonic development, such as social interactions between reproductives and non-reproductive castes, are also determinant force for the neotenic differentiation (Lüscher, 1974; Shimoji et al., 2017; Sun et al., 2017; Masuoka et al., 2021). Recently, neotenic differentiation was studied by applying induction methods in species with bifurcated and linear caste differentiation pathways (Saiki and Maekawa, 2011; Shimoji et al., 2017), focusing on morphogenetic processes during differentiation and the underlying physiological regulation (Saiki et al., 2015; Oguchi and Miura, 2019; Oguchi et al., 2020). By reviewing accumulated knowledge on the physiological and developmental regulation of neotenic differentiation in termites, the evolutionary processes toward the acquisition of neotenic reproductives are hypothesized.

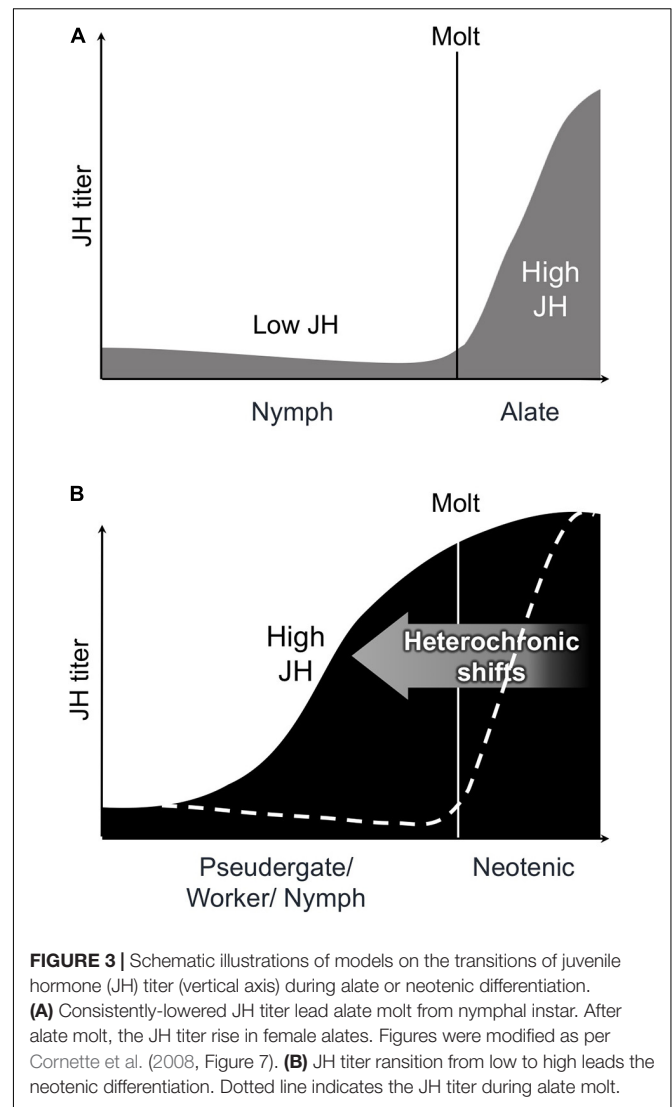
## MODULARITY: REPRODUCTIVE ORGAN-SPECIFIC MORPHOGENESIS

Neotenic reproductives in termites exhibit the juvenile characteristics of hemimetabolous insects, and thus, they are termed “neotenic” (Thorne, 1996). Meanwhile, they lack imaginal characteristics such as well-developed wings and compound eyes (Maekawa et al., 2008; Saiki and Maekawa, 2011). Neotenic can be roughly classified into two types: “nymphoids” that differentiate from nymphs and “ergatoids” that differentiate from workers (Korb and Hartfelder, 2008). Depending on the developmental stages from which neotenic derive, the developmental degree of imaginal characters differs (e.g., nymphoids have wing buds and slightly developed eyes, whereas ergatoids lack these features). However, in all cases, neotenic possess well-developed gonads that are comparable to those of primary reproductives (Thorne, 1996; Korb and Hartfelder, 2008; Saiki and Maekawa, 2011; Oguchi et al., 2016). This suggests that gonadal development is accelerated in neotenic in contrast to other body parts. This acceleration might be a case of “paedomorphosis,” which is a type of heterochrony that involves a delay of specific organ development (Reilly et al., 1997; Nalepa and Bandi, 2000). The adaptive significance of neotenic differentiation, in which only some imaginal characters develop, might be that it permits individuals to immediately become reproductives through fewer moltings. In most cases, one molting event is required for neotenic differentiation from the previous undifferentiated stages, such as pseudergates, workers, or nymphs (Korb and Hartfelder, 2008), except in some species with bifurcated caste differentiation pathways of *Reticulitermes labralis*, *Nasutitermes corniger*, and *Nasutitermes aquilinus*, in which the pre-neotenic stages and two molts are required (Thorne and Noirot, 1982; Su et al., 2017; da Silva et al., 2019). The fact that the neotenic differentiation requires moltings suggests that morphological alterations are required for reproductive activities such as copulation and oviposition. In the case of neotenic molt (only female individuals) in a dampwood

termite *Hodotermopsis sjostedti*, which possesses a linear caste differentiation pathway, the loss of styli at the abdominal tip and expansion of the seventh sternites are observed (Oguchi and Miura, 2019). Therefore, the differentiation of neotenic reproductives may be accomplished by a partial release of immature individuals from arrested development, particularly in the reproductive organs. In other words, the pre-existing imaginal developmental processes (i.e., a late differentiation processes) are partially involved in neotenic differentiation; therefore, neotenic differentiation may be regarded as a case of “modular heterochrony.” Thus, the neotenic reproductive is a distinctive phenotype (caste) that morphologically differs from primary reproductives (alates) (Figure 2). These mosaic-like phenotypes were not only observed in termites but also in eusocial hymenopteran species, particularly in ants (Wheeler and Nijhout, 1981; Abouheif and Wray, 2002; Miyazaki et al., 2010; Yang and Abouheif, 2011). Of note, “ergatoid queen” in ants, which has both the queen and worker traits, was believed to have emerged from the combination of the caste developmental systems (Miyazaki et al., 2010; Yang and Abouheif, 2011; Molet et al., 2012). Therefore, modular and heterochronic regulation by tinkering with the pre-existing developmental systems are thought to be important and ubiquitous evolutionary mechanisms for the acquisition of novel castes (cf. Cohen, 1999).

## HETEROCHRONIC SHIFTS VIA PHYSIOLOGICAL CONTROL

Generally in insects, molting and metamorphosis are coordinated by JH and molting hormone (i.e., ecdysone) (Belles and Santos, 2014). It is well known that the imaginal molt is induced by the lowering of JH titers in an insect (Romaña et al., 1995; Treiblmayr et al., 2006). A similar physiological pattern is also present during alate differentiation in termites (Figure 3A; Nijhout and Wheeler, 1982; Cornette et al., 2008). However, detailed physiological analyses, especially on JH dynamics, have yet to be conducted concerning the differentiation into neotenic reproductives, although previous studies predicted that the shift from low to high JH titers during the intermolt period would induce neotenic differentiation (reviewed in Nijhout and Wheeler, 1982; Korb, 2015). Some studies actually reported an extraordinary high JH titer in the female neotenic (e.g., Elliott and Stay, 2007; Maekawa et al., 2010; Korb et al., 2012; Saiki et al., 2015). Similar tendencies of high JH titers were observed after female alate differentiation (Cornette et al., 2008; Maekawa et al., 2010). The high JH titers in female reproductives probably contribute to the development of female reproductive systems, including ovaries (Korb, 2015; Saiki et al., 2015). Furthermore, in many insects including termites, JH is sequestered in fat bodies and engaged in yolk protein synthesis (Nijhout, 1994), suggesting that this function of JH is also driven at the time of neotenic differentiation. However, the JH function in the ovarian development is only applicable to the differentiation of female neotenic, and thus, the JH action leading to the differentiation of male neotenic should also be elucidated in future studies.



Recently, the physiological regulation at the time of neotenic differentiation was unraveled in the dampwood termite *H. sjostedti* (Oguchi et al., 2020). This research applied an induction method for neotenic differentiation in which the sex caste ratio was artificially manipulated (Shimoji et al., 2017). Under these experimental conditions, the existence of only a male (or female) neotenic reproductive promotes the differentiation of female (or male) neotenic from pseudergates. Their findings illustrated that the JH titer of female pseudergates in the presence of a male neotenic without a female neotenic continuously lowered and then increased immediately before the molt (Oguchi et al., 2020). Thus, this study supports the hypothesis by Nijhout and Wheeler (1982), at least in terms of the female neotenic differentiation, considering that neotenic differentiation requires a transition of JH titers from low to high (Figure 3B). The similar tendencies and models have been shown in other species, such as *R. speratus* (Saiki et al., 2015) and *Cryptotermes secundus* (Korb et al., 2012), suggesting that this model can be applicable to other

termite lineages, at least lower termite species. Moreover, the JH analog application of female pseudergates inhibits the reproductive organ development and neotenic molt (Oguchi et al., 2020). This role of JH is similar to the antimetamorphic action of insects and lowering JH titer, which are required for the imaginal molts (Nijhout, 1994). Therefore, the fact that female neotenic differentiation requires low JH titers suggests that the neotenic molt can be regarded as a type of imaginal molt and rapid change of JH titers might be heterochronic shifts (**Figures 3A,B**). In other words, the neotenic reproductive can be regarded as a “physiological imago.” Recently, the master regulatory pathway controlling molting and metamorphosis, i.e., the MEKRE93 or Met-Krh1-E93 pathway, was demonstrated to work downstream of JH and ecdysone in insects (Belles and Santos, 2014; Ureña et al., 2014), suggesting that this pathway is also involved in caste regulation in termites (Korb and Belles, 2017; Miura and Maekawa, 2020).

## EVOLUTIONARY IMPLICATIONS

One of the most important steps in the evolution of eusociality is the acquisition of reproductive labor division (Wilson, 1971; Lin and Michener, 1972). In termites, especially in basal species with linear caste pathways, immature individuals, that are homologous to “nymphs” in general hemimetabolous insects, engage in colony tasks without participating in reproduction (Korb and Hartfelder, 2008). These immature individuals undergo successive moltings, but they do not completely develop adult-specific characteristics such as compound eyes and wings (Miura et al., 2004; Katoh et al., 2007; Nii et al., 2019). Considering the evolutionary process of termite eusociality, the appearance of immature castes that are engaged in colony tasks (i.e., pseudergates) would be an important step (cf. Nalepa and Bandi, 2000). Before the emergence of such immature castes, bifurcation (i.e., develop or less develop) of imaginal organ development within the colony members is suggested to lead the reproductive division of labor (Bourguignon et al., 2016). Therefore, during the eusocial evolution in termites, it is suggested that the postembryonic development of hemimetabolous insects is partially (i.e., body parts specifically) or completely arrested during the linear adult developmental process (Bourguignon et al., 2016).

Thus, what mechanisms enable neotenic wingless reproductive differentiation? Some studies provided circumstantial evidences that the development of adult-specific characteristics (e.g., reproductive organs, wings, compound eyes, sensillae) is connected and induced under the control of physiological signals (i.e., hormonal signals), leading to alate differentiation (e.g., Miura and Matsumoto, 1996). Therefore, during neotenic differentiation, such alate-specific characteristics could also be expressed. In fact, neotenic reproductives display dark coloration of the cuticles caused by pigmentation and sclerotization during differentiation that resembles alate coloration (e.g., Watson and Abbey, 1985; Hu and Forschler, 2012). Moreover, it was reported

that ergatoids possessed wing bud-like structures on their thoraces and slightly developed compound eyes on their heads (e.g., Noirot and Thorne, 1988; Miura and Matsumoto, 1996; da Silva et al., 2019). This phenomenon suggests that the development of these adult-specific characteristics is interlinked, permitting their expression simultaneously with the gonadal development required for the differentiation of functional ergatoids. These links among adult-specific characteristics are assumed to arise from the linkage of gene regulatory networks.

Recently, transcriptomic analysis suggested that Ras-MAPK pathway genes involved in ergatoid differentiation in *Reticulitermes labralis* (Ye et al., 2019). In general, the Ras-MAPK pathway is known to control the signal transduction from plasma membrane to nucleus, regulating downstream genes involved in cell proliferation, differentiation, and cell death (Foster and Malek, 2016). Therefore, specific regulations in such cellular processes may be crucial for ergatoid differentiation. It is unclear whether these gene expression changes also occur during nymphoid differentiation; however, the differences of gene expression patterns between ergatoids and nymphoids may help to understand the genetic networks that regulate body part-specific development.

As mentioned previously, there are several morphological and functional differences between alates and neotenic, and thus, there should be different physiological regulatory mechanisms underlying the differentiation processes leading to the two reproductive types. In both alate and neotenic differentiation, JH and ecdysone pathways might be involved in each molt, although the timing of JH elevation differs between them. Therefore, these gaps might explain the partial differences between alates and neotenic. During neotenic differentiation, only some body parts and organs that are related to reproduction undergo further developmental processes that also occur during alate differentiation (**Figure 2**). In soldier differentiation in termites, such body part-specific morphogenesis, i.e., mandibular enlargement (Koshikawa et al., 2003; Watanabe and Maekawa, 2008; Watanabe et al., 2014; Miura and Maekawa, 2020), is organized by patterning genes providing spatial information, such as Hox genes, which are upregulated under the control of hormonal factors (e.g., Toga et al., 2013; Sugime et al., 2019). Considering these mechanisms, neotenic differentiation should also require similar regulatory mechanisms of gene expression including toolkit genes such as Hox genes and hormonal factors. Obviously, because anterior body parts are dramatically modified during soldier differentiation whereas posterior body parts such as reproductive traits develop during neotenic differentiation, different Hox genes should be responsible for the body-part development in each caste. During the evolutionary processes of the termite lineage, it is suggested that such regulatory mechanisms leading to body part-specific differentiation processes were acquired in the common ancestor, resulting in the appearances of novel castes, such as soldiers and neotenic reproductives. Thus, comparative approaches between termites and sister-group cockroaches, the genus *Cryptocercus*, may uncover the underpinnings of social evolution in termites.

## AUTHOR CONTRIBUTIONS

KO, KM, and TM wrote the manuscript. All authors read and approved the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by a Grant-in-Aid for Scientific Research A (No. 25251041) for TM and a Grant-in-Aid for JSPS

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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