Hormonal Signaling in Cnidarians: Do We Understand the Pathways Well Enough to Know Whether They Are Being Disrupted?

Running title: Hormonal signaling in cnidarians

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Abstract

Cnidarians occupy a key evolutionary position as basal metazoans and are ecologically important as predators, prey and structure-builders. Bioregulatory molecules (e.g., amines, peptides and steroids) have been identified in cnidarians, but cnidarian signaling pathways remain poorly characterized. Cnidarians, especially hydras, are regularly used in toxicity testing, but few studies have used cnidarians in explicit testing for signal disruption. Sublethal endpoints developed in cnidarians include budding, regeneration, gametogenesis, mucus production and larval metamorphosis. Cnidarian genomic databases, microarrays and other molecular tools are increasingly facilitating mechanistic investigation of signaling pathways and signal disruption. Elucidation of cnidarian signaling processes in a comparative context can provide insight into the evolution and diversification of metazoan bioregulation. Characterizing signaling and signal disruption in cnidarians may also provide unique opportunities for evaluating risk to valuable marine resources, such as coral reefs.

Hormonal signaling in cnidarians has not been fully characterized on either a molecular or a biochemical level, and the potential for signal disruption is largely unknown. This review considers the state of knowledge on hormonal signaling, chemical bioregulation and signal disruption in cnidarians, focusing particularly on advances since the publication of the results of the EDIETA (Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment) workshop (deFur et al., 1999).

Why cnidarians: diversity, evolutionary significance and ecological role

The phylum Cnidaria contains four extant classes, the Hydrozoa (e.g., hydras), Scyphozoa ("true" jellyfishes), Cubozoa (box jellies) and Anthozoa (e.g., corals and anemones). The number of cnidarian species has not been rigorously assessed, but is estimated to be around 10,000 (Brusca and Brusca, 1990). A definitive cnidarian species inventory is currently being established (D.G. Fautin, personal communication, December 30, 2005), and progress to date indicates that the Subclass Hexacorallia (that includes anemones and corals) alone contains 2899 valid species (Fautin, 2005).

Cnidarians are usually classified as basal metazoans that form a sister group to the bilaterians, although these ancient evolutionary relationships are a subject of active research (Holland, 1998; Gröger and Schmid, 2001; Martindale, 2005; Rokas et al., 2005). Because of their basal position and simple organization, it is sometimes assumed that bioregulation of cnidarian physiology must also be simple and that vertebrate features absent from model protostomes are likely to have evolved after the divergence of the protostomes and the deuterostomes. Somewhat surprisingly, a comparative study indicated that more than ten percent of genes shared between cnidarians and humans have

apparently been lost from model protostomes (Kortschak et al., 2003). Thus, it is not possible to use conserved features between vertebrates and model invertebrates alone to predict the complexity of cnidarian signaling processes (Miller et al., 2005; Technau et al., 2005). Further, elucidation of cnidarian signaling processes in a comparative context can provide insight into the evolution and diversification of metazoan bioregulation.

In addition to their key evolutionary position, cnidarians are also important as both predators and prey in aquatic ecosystems. Gelatinous cnidarians serve as prey for diverse taxa including other cnidarians, fishes and turtles. While gelatinous cnidarians are sometimes regarded as relatively unpalatable or of low nutritional value (e.g., Avent et al., 2001), cnidarians may be widely underestimated as prey sources because they tend to be rapidly digested (Arai et al., 2003; Arai, 2005). As predators, cnidarians can dramatically change the composition of plankton and may impair fisheries yield through dietary overlap and direct predation (Arai, 1988; Purcell and Arai, 2001; Purcell and Sturdevant, 2001). As an example, the hydroid, *Clytia gracilis*, can remove up to 40% of copepod nauplii daily production in a region of the Georges Bank (Atlantic coast of the United States, Madin et al., 1996). Copepod nauplii are a primary food source for larval cod and haddock, so hydroids can significantly reduce food availability and may also prey directly on the larval fish (Madin et al., 1996). Suspension-feeding benthic cnidarians also impact planktonic composition and may play an important role in benthic-pelagic coupling. For example, a Mediterranean gorgonian was found to consume large numbers of eggs and invertebrate larvae, particularly larval bivalves, with unknown effects on bivalve recruitment and population dynamics (Rossi et al., 2004).

Jellyfish (medusae, which may be hydrozoan, scyphozoan or cubozoan) aggregations or blooms are common episodic events, and the frequency and extent of blooms may be increasing (Mills, 2001). Jellyfish blooms can have major ecological consequences. For example, an exotic jellyfish recently accounted for 98% of total fisheries sampling in the Yangtze estuary in China (summarized by Xian et al., 2005). Jellyfish blooms are caused and maintained by a combination of physical and poorly understood behavioral and physiological processes (Lotan et al., 1994; Purcell et al., 2000; Graham et al., 2001; Lucas, 2001). Jellyfish blooms may also be aggravated by anthropogenic factors (Mills, 2001) including overfishing of competitors (Brodeur et al., 2002), eutrophication and changes in estuarine circulation (Xian et al., 2005). The role of hormonal or pheromonal signals in regulating jellyfish blooms is unknown. Similarly, it is unknown whether environmental chemicals affect jellyfish signaling and bloom dynamics.

Cnidarians also provide three-dimensional structure to benthic ecosystems, most notably tropical coral reefs. Reef ecosystems provide habitat for diverse taxa, protect shorelines and provide commercial and recreational resources for humans. Scleractinian coral biology is of particular interest due to the importance of corals in maintaining tropical reefs and increasing human concerns about reef degradation (Gardner et al., 2003; Hughes et al., 2003; Pandolfi et al., 2003). The chemical signals that regulate coral gametogenesis and spawning are poorly understood, and it is unknown whether environmental chemicals may disrupt these signals.

Routes of exposure

Cnidarians may be exposed to chemicals through uptake of dissolved compounds, ingestion of food particles, or contact with suspended solids and sediments. The relative importance of these routes of exposure varies with life history parameters, particularly habitat and mode of nutrition. Adult cnidarians may be benthic or pelagic, cnidarian gametes may be brooded or spawned, and larvae spend varying times (hours to months) in the water column. Cnidarians may be entirely carnivorous or may contain algal symbionts that fill some or all of their energetic needs. Exposure of scleractinian corals to chemicals is of particular concern because reef ecosystems are often located near highly populated coasts and may be exposed to contaminants through riverine input, sewage effluent, run-off, groundwater discharge, marine activities and spills (reviewed by Peters et al., 1997).

Cnidarians can take up dissolved chemicals through diffusion. The freshwater *Hydra spp.* are commonly used (Blaise and Kusui, 1997; Holdway et al., 2001; Pascoe et al., 2003) and other cnidarians are occasionally used (e.g., anemones Mercier et al., 1997; colonial hydroids Chicu et al., 2000) in toxicity testing with dissolved contaminants. One explanation for the observed sensitivity of hydras to a range of chemicals is their diploblastic organization, which allows all cells potentially to be exposed to the surrounding medium and associated contaminants (Ingersoll et al., 1999). Similarly, Peters et al. (1997) suggested that reef-building corals might be particularly sensitive to lipophilic contaminants because corals typically possess thin lipid-rich tissues, facilitating direct uptake. Incubations with radiolabeled compounds have demonstrated that corals can remove estrogens (Tarrant et al. 2000), petroleum hydrocarbons (Solbakken et al. 1984), and benzo[a]pyrene (Kennedy et al. 1992) from the surrounding seawater.

Few studies have examined the bioavailability of particle-associated contaminants to cnidarians. Harter and Matthews (2005) suggested that the burrowing anemone *Nematostella vectensis* could be used in sediment toxicity testing. Mayer et al. (2001) compared liquid removed from the oral cavity of a sea anemone with other invertebrate digestive fluids for their capacity to solubilize radiolabeled benzo[a]pyrene and zinc. They concluded that the material extracted from the anemone had a low ability to solubilize sediment-associated contaminants, but the method of sampling may not have accurately reflected the actual capacity of the anemone to solubilize contaminants. For example, particles may stick to the mucus-lined interior of the oral cavity and be subject to enzymatic activity.

Cnidarians may also be exposed to contaminants through ingestion of prey, although few studies have tested the effects of dietary exposures. For example, aqueous or dietary exposure of sea anemones to tributyltin (TBT) can affect zooxanthallae abundance, associated bacteria, and nematocyst discharge (Mercier et al., 1997). While this study was relatively unique in comparing an aqueous and a dietary exposure, only a single concentration was tested in each case and accumulation of TBT in the anemones was not measured. Clearly additional quantitative studies are needed to test the relative importance of different routes of chemical exposure to cnidarians.

In addition to directly affecting cnidarians, chemicals may also accumulate in cnidarian tissues, where they may affect cnidarian predators. Cnidarian tissues can be very lipid rich (Stimson, 1987), allowing for rapid uptake and storage of lipophilic contaminants. For example, high levels of PCBs were measured in tissues of a soft coral from Guam, relative to other invertebrates. Tissue accumulation of PCBs may be due in part to the

high tissue concentration of triglycerides and other lipids (Denton et al., 2006). While

uptake of lipophilic chemicals by cnidarians may be rapid, metabolism and elimination

often proceeds slowly (Solbakken et al., 1984; Kennedy et al., 1992; Peters et al., 1997),

possibly due to low activity of xenobiotic metabolizing enzymes (Gassman and Kennedy,

1992). The tissue lipid concentration of some corals decreases following the release of

eggs or larvae (Stimson, 1987); however, additional study is needed to determine whether

accumulated lipophilic contaminants are mobilized during spawning events. In addition

to organic chemicals, heavy metals can also accumulate in cnidarian tissues (Mitchelmore

et al., 2003) and can be transferred up the food chain. For example, high levels of

cadmium and other metals in leatherback turtles are thought to be derived from their

jellyfish prey (Caurant et al., 1999).

Hormonal Signaling in Cnidarians

In classical endocrinology, hormones are synthesized by endocrine organs, secreted

into the bloodstream and transported to target tissues where they bind to specific

receptors. Cnidarian cells are differentiated into tissues but not into specialized endocrine

organs, and circulation occurs primarily through diffusion. While the term

"endocrinology" is not strictly applicable to cnidarians, this discussion may be extended

to include physiological regulation controlled by bioactive signal molecules (e.g.,

secondary messengers, neurotransmitters and hormones) synthesized within the

organism. This section will briefly describe cnidarian hormones and neuropeptides and

recent discoveries in cnidarian signaling. For a more extensive discussion of the cnidarian

bioregulation, readers can consult several recent reviews (Leitz, 2001; Grimmelikhuijzen et al., 2002; Müller and Leitz, 2002; Tarrant, 2005).

Cnidarians are among the simplest animals to possess a nervous system. The cnidarian nervous system has been described as a "nerve net" but may also contain nerve rings and other areas of centralization (Grimmelikhuijzen, 1985). Cnidarians synthesize monoamines that act as neurotransmitters in other animals including catecholamines and indolamines (Pani and Anctil, 1994; Leitz, 2001). Indolamines, such as serotonin, may act as neurohormones in some cnidarians. In the sea pansy, *Renilla koellikeri*, serotonin stimulates rhythmic muscular contraction (Anctil, 1989) and spawning (Tremblay *et al.* 2004). Seasonal peaks in melatonin coincided with the first stages of sexual maturation (Mechawar and Anctil, 1997). Serotonin can also experimentally induce metamorphosis of hydrozoan larvae (McCauley, 1997; Walther and Fleck, 1998). Specific binding sites have been identified in cnidarians for serotonin (Hajj-Ali and Anctil, 1997), β-adrenergic agonists (Awad and Anctil, 1993) and γ-aminobutyric acid (GABA, Pierobon et al., 1995). Also, genes for G protein-coupled receptors have been cloned (Nothacker and Grimmelikhuijzen, 1993; New et al., 2000; Bouchard et al., 2003), but the ligands for these receptors have not yet been identified. One G protein-coupled receptor is phylogenetically related to dopamine and adrenergic receptors but not responsive to known biogenic amines (Bouchard et al., 2003; Bouchard et al., 2004).

The majority of signaling molecules known in cnidarians are peptides that may act as neurotransmitters and/or neurohormones. Neuropeptides have been extensively studied as regulators of hydrozoan development and metamorphosis, and hundreds of peptides are systematically being purified, sequenced and screened from *Hydra magnipapillata*

(Takahashi et al., 1997). The "head activator" peptide, first purified and sequenced from

Hydra attenuata and *Anthopleura elegantissima* is a potent morphogen that triggers

neurogenesis and head-specific growth (Schaller and Bodenmüller, 1981).

Immunoreactive gonadotropin-releasing hormone (GnRH) has been detected in

cnidarians and may help to regulate gametogenesis and spawning (Anctil, 2000; Twan et

al., 2006). Peptides are roughly classified into families based on the amino acid sequence

of the C-terminus (e.g., [G]LW-amides and RF-amides), and in some cases peptides with

a similar C-terminus seem to have similar biological activity (Leitz, 2001). LW-amides,

including "metamorphosin A" stimulate contraction of some cnidarian muscles and

trigger metamorphosis of hydrozoan planulae (Leitz et al., 1994; Takahashi et al., 1997;

Plickert et al., 2003). While their role in cnidarian physiology has not been fully

elucidated, LW-amides have been hypothesized to function in tentacle formation,

metamorphosis and larval physiology (McFadden and Spencer, 1991; Brumwell and

Martin, 1996). RF-amides affect cnidarian muscle contraction, feeding responses and

exfoliation of gamete follicles (McFarlane and Grimmelikhuijzen, 1991; Pierobon et al.,

1997b; Tremblay et al., 2004). The receptors for these peptides have not been identified,

but genes encoding preprohormones have been sequenced from several species (Leviev et

al., 1997; Darmer et al., 1998).

Vertebrate-type sex steroids have been detected by immunoassay in several

anthozoans, and concentrations vary seasonally and between sexes (Slattery et al., 1999;

Tarrant et al., 1999; Pernet and Anctil, 2002; Twan et al., 2003). Most notably, estrogen

concentrations were elevated in tissues of the coral *Euphyllia ancora* (Twan et al., 2003)

and the sea pansy *Renilla koellikeri* prior to spawning (Pernet and Anctil, 2002), and

testosterone concentration in the soft coral *Sinularia polydactyla* decreased following

spawning (Slattery et al., 1997). Estradiol is released into the water column in association

with anthozoan spawning (Atkinson and Atkinson, 1992; Slattery et al., 1999; Twan et

al., 2003). Together these observations prompted speculation that free or conjugated

estrogens might serve as chemical cues or pheromones to stimulate coral spawning, but

the source of sex steroids in cnidarian tissues has not been demonstrated. Low levels of

estrogen synthesis from androgen substrates by coral homogenates has been reported in

one study (Twan et al., 2003), but other studies have not detected estrogen synthesis

(Slattery et al., 1997; Tarrant et al., 2003). The mechanism of steroid hormone action in

cnidarian tissues is also unknown. While steroid receptors have not been identified in any

cnidarian, several cnidarian nuclear receptor genes have been identified, including

homologs to COUP-TF (chicken ovalbumin upstream promoter transcription factor,

Escriva et al., 1997; Grasso et al., 2001; Tarrant, Unpublished data) and RXR (retinoic

acid "x" receptor, Kostrouch et al., 1998). Some cnidarian nuclear receptors are

sufficiently divergent from vertebrate sequences that they cannot easily be assigned to a

nuclear receptor subfamily or a clear vertebrate homolog (Grasso et al., 2001). Because a

classical estrogen receptor has not been identified in any cnidarian, estrogens or other

steroids may act through a "functional estrogen receptor" that does not belong to the

steroid receptor protein family described in vertebrates.

Overall, hormonal signaling and bioregulatory pathways are still largely

uncharacterized in cnidarians. Considerable progress has been made in identifying

bioactive molecules, particularly peptides, but in most cases their receptors have not been

identified and their precise physiological function is unknown. Other classes of signaling

molecules such as fatty acids and eicosanoids may also act as cnidarian hormones (Coll et al., 1994; Coll et al., 1995; Pierobon et al., 1997b; Pierobon et al., 1997a), but more research is needed to identify the functions of these compounds and their mechanisms of action. Iodinated organic molecules, such as thyroxine, have been proposed to hormonally regulate strobilation and metamorphosis in the scyphozoan jellyfish *Aurelia aurita* (Spangenberg, 1971). A recent study indicates that this physiological response is actually induced by iodine, which is formed through oxidation of iodide (Berking et al., 2005). The authors concluded that thyroxine is a waste product in this pathway that later developed as a vertebrate metabolic hormone.

Endocrine Disruption in Cnidarians

Endocrine disruption has not been documented in cnidarians, which is not surprising given that cnidarian hormonal signaling pathways are poorly characterized and few appropriate endpoints have been established. A few studies have tested the effects of estrogenic compounds on cnidarians. *Montipora capitata* coral colonies treated with exogenous estradiol (nominally 2.3 µg L^{-1}) for three weeks prior to spawning released fewer (29%) egg-sperm bundles than control colonies (Tarrant et al., 2004). *Porites compressa* coral fragments exposed continuously to 2 ng L^{-1} estrone for 2 - 8 weeks had lower (13 - 24%) skeletal growth rates than controls (Tarrant et al., 2004). On the other hand, when *Hydra vulgaris* was exposed to ethinyl estradiol and bisphenol A, both of which are estrogenic in vertebrates, adverse physiological effects were only seen at high doses (> 40 µg L^{-1}) (Pascoe et al., 2002). Bisphenol A also impaired sexual and asexual

reproduction in *Hydra oligactis*, but the authors concluded that the adverse effects were most likely due to general toxicity of the compound (Fukuhori et al., 2005).

Cnidarians, particularly hydras, are widely used in toxicity testing and in teratogenicity testing of pharmaceuticals. Several compounds that disrupt endocrine signaling in other animals have been tested for toxicity or disruptive activity in cnidarians. For example, reef-building corals are affected by herbicides such as atrazine and diuron, but the primary effects seem to be direct toxicity to photosystem II of the symbiotic algae within the coral tissues (reviewed by Jones, 2005). Cnidarians are sensitive to metals, including cadmium, zinc and butyltin (Chicu and Berking, 1997; Mercier et al., 1997; Holdway et al., 2001; Harter and Matthews, 2005), although reproductive abnormalities comparable to the imposex phenomenon observed in gastropods have not been noted.

Toxic effects of petroleum products and dispersants have also been observed, particularly on coral larval metamorphosis (Epstein et al., 2000; Negri and Heyward, 2000). A suite of organic compounds, including hydrocarbons and tributyltin, antagonize induced metamorphosis in the colonial hydroid *Hydractinia echinata*. Structure-activity relationships have been determined for several classes of compounds with respect to their effect on *H. echinata* metamorphosis (Chicu and Berking, 1997; Chicu et al., 2000). Generally, and particularly within a class of compounds, inhibition of metamophosis increases with increasing octanol/water partition coefficient (i.e., more lipophilic compounds are more inhibitory).

As described in the previous section, considerable progress has been made recently toward identifying bioregulatory molecules, especially peptides that regulate

metamorphosis. A mechanistic understanding of signaling processes in cnidarians will allow for monitoring of signal disruption and for a better distinction between general toxicity and signal disruption.

Test Species and Endpoints

While few studies have explicitly tested for disruption of hormonal signaling, several cnidarian taxa have been used recently in toxicity testing, including hydras, colonial hydroids, sea anemones, and scleractinian corals. In general, the relative sensitivity of different cnidarians to chemicals has not been assessed. One comparative study demonstrated that *Hydra vulgaris* is more sensitive to cadmium and zinc toxicity than *Hydra viridissima* (Holdway et al., 2001).

Hydras have been the cnidarians most widely used in toxicity testing (Blaise and Kusui, 1997; Holdway et al., 2001; Pascoe et al., 2003). While toxicity testing with hydras has not been standardized, the most common sublethal endpoints are budding, polyp structure and polyp regeneration. Most laboratory strains of hydras reproduce primarily by budding, but sexually reproducing strains have been used to test the effects of chemicals on gametogenesis (Segner et al., 2003; Fukuhori et al., 2005). *Hydra vulgaris* was selected as the cnidarian test species in the European IDEA project, which investigated effects of environmental estrogens on diverse invertebrates (Segner et al., 2003). As test species, hydras have the advantages of rapid growth, small size, ease of culture, and the availability of clonal strains. In addition, the *Hydra magnipapillata* genome is being sequenced, and a wealth of information is available regarding hydra development, neurobiology and toxicology. One disadvantage of using hydras to screen

for disruption of cnidarian signaling is that hydras are highly derived freshwater animals and not necessarily representative of the predominantly marine, more "typical" cnidarians.

In addition to solitary hydras, colonial marine hydrozoans in the genus *Hydractinia* are widely used in studies of development, metamorphosis and immune response (reviewed by Frank et al., 2001). *Hydractinia spp.* have been particularly useful in mechanistic studies linking an environmental stimulus (compound(s) derived from bacterial films) with a signaling cascade resulting in metamorphosis (reviewed by Müller and Leitz, 2002). While *Hydractinia spp.* share many advantages with hydras and have been used to evaluate the effects of some chemicals (Leitz and Wirth, 1991), they have not been fully exploited as a model for investigation of toxicity and signal disruption by environmental contaminants.

Anemones have not been used extensively in toxicity testing to date but may be used more extensively in the future. The small burrowing anemone, *Nematostella vectensis,* is easy to maintain, matures rapidly, and spawns predictably in the laboratory (Darling et al., 2005). The *N. vectensis* genome has been sequenced and assembled (Sullivan et al., 2006), and characterization of *N. vectensis* genes has already provided significant insight into the evolution of animal development. *N. vectensis* has a wide geographic range, including large portions of the Pacific and Atlantic coasts of North America and the Southeastern coast of England. *N. vectensis* is most common on the Atlantic coast of the United States, where it inhabits a range of estuarine habitats and may be useful as an ecotoxicological model. Harter and Matthews (2005) exposed *N. vectensis* to acute and chronic doses of cadmium chloride and measured mortality, change in weight, and egg

production. They found that the sensitivity of *N. vectensis* to cadmium is similar to the

sensitivity of other invertebrates and suggested that *N. vectensis* could be further

developed as a test organism for dissolved and sediment-associated toxicity testing. Other

anemones have been used in toxicity testing; for example, exposure of *Aiptasia pallida* to

tributyltin resulted in changes in mucus secretion and abundance of symbionts, bacterial

aggregates and undischarged nematocysts (Mercier et al., 1997).

Scleractinian corals have been recently used to evaluate the effects of chemicals

including herbicides, petroleum products, dispersants and estrone (Epstein et al., 2000;

Negri and Heyward, 2000; Tarrant et al., 2004; Jones, 2005). Endpoints measured

included tissue thickness, skeletal growth, fecundity, fertilization, and larval

metamorphosis. As test species, scleractinian corals have clear ecological relevance but

are not easily accessible to many laboratories due to geographic constraints and legal

protections. Relative to hydras or *Nematostella,* corals are difficult to maintain in the

laboratory and generally take several years to reach reproductive maturity. Analysis of

reproductive endpoints is further limited by the seasonal reproductive cycle of many

corals.

Molecular tools such as cDNA arrays (Edge et al., 2005; Morgan et al., 2005) and the

ELISA-based "Cellular Diagnostics" (which measures concentrations of proteins

associated with detoxification and stress responses, Downs et al., 2005) have been

developed for corals and may be used to evaluate the effects of chemicals. Differential

display has been effectively used to screen for bioactivity of peptides in hydras

(Takahashi et al., 1997), and this approach could be adapted to screen for signal

disruption. Collectively, these techniques can provide molecular or biochemical

endpoints for chemical effects on cnidarian physiology, including disruption of signaling

pathways.

The future of cnidarians as indicators of endocrine disruptive chemicals

Given the limited knowledge of cnidarian signaling pathways and the few studies of

signal disruption, cnidarians currently have limited use as models for evaluating signal

disruption. On the other hand, cnidarians are sensitive to many stressors and have been

used as indicators of water quality. Thus, with a better understanding of regulatory

processes and development of appropriate endpoints, cnidarians may become valuable

indicators of exposure to disruptive chemicals and other stressors. In many cases,

cnidarians are subjected to multiple stressors, which complicates the detection of signal

disruption by environmental contaminants in the field. For example, in coastal

environments, cnidarians and other organisms may simultaneously be exposed to

increased sedimentation, eutrophication, bacterial loads and mixtures of potentially

disruptive chemicals. Without a mechanistic understanding of cnidarian regulatory

pathways, it will be extremely difficult to separate signal disruption from other stresses.

Ongoing studies, facilitated by the release of genomic sequences and the development of

molecular tools will provide a more complete context for evaluating disruption of

cnidarian signaling.

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