Indomethacin reproducibly induces metamorphosis in *Cassiopea xamachana* scyphistomae

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Cassiopea xamachana jellyfish are an attractive model system to study metamorphosis and/or cnidarian-dinoflagellate symbiosis due to the ease of cultivation of their planula larvae and scyphistomae through their asexual cycle, in which the latter can bud new larvae and continue the cycle without differentiation into ephyrae. Then, a subsequent induction of metamorphosis and full differentiation into ephyrae is believed to occur when the symbionts are acquired by the scyphistomae. Although strobilation induction and differentiation into ephyrae can be accomplished in various ways, a controlled, reproducible metamorphosis induction has not been reported. Such controlled metamorphosis induction is necessary for an ensured synchronicity and reproducibility of biological, biochemical and molecular analyses. For this purpose, we tested if differentiation could be pharmacologically stimulated as in *Aurelia aurita*, by the metamorphic inducers thyroxine, KI, NaI, lugol's iodine, H_2O_2, indomethacin, or retinol. We found reproducibly induced strobilation by 50 µM indomethacin after 6 days of exposure, and 10-25 µM after 7 days. Strobilation under optimal conditions reached 80-100% with subsequent ephyrae release after exposure. Thyroxine yielded inconsistent results as it caused strobilation occasionally, while all other chemicals had no effect. Thus, indomethacin can be used as a convenient tool for assessment of biological phenomena through a controlled metamorphic process in *C. xamachana* scyphistomae.

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Abstract

Cassiopea xamachana jellyfish are an attractive model system to study metamorphosis and/or cnidarian-dinoflagellate symbiosis due to the ease of cultivation of their planula larvae and scyphistomae through their asexual cycle, in which the latter can bud new larvae and continue the cycle without differentiation into ephyrae. Then, a subsequent induction of metamorphosis and full differentiation into ephyrae is believed to occur when the symbionts are acquired by the scyphistomae. Although strobilation induction and differentiation into ephyrae can be accomplished in various ways, a controlled, reproducible metamorphosis induction has not been reported. Such controlled metamorphosis induction is necessary for an ensured synchronicity and reproducibility of biological, biochemical and molecular analyses. For this purpose, we tested if differentiation could be pharmacologically stimulated as in *Aurelia aurita*, by the metamorphic inducers thyroxine, KI, NaI, lugol's iodine, H_2O_2, indomethacin, or retinol. We found reproducibly induced strobilation by 50 µM indomethacin after 6 days of exposure, and 10-25 µM after 7 days. Strobilation under optimal conditions reached 80-100% with subsequent ephyrae release after exposure. Thyroxine yielded inconsistent results as it caused strobilation occasionally, while all other chemicals had no effect. Thus, indomethacin can be used as a convenient tool for assessment of biological phenomena through a controlled metamorphic process in *C. xamachana* scyphistomae.

Keywords: *Cassiopea xamachana*, chemical inducer, indomethacin, strobilation, scyphistomae.

Introduction

Cnidarian-dinoflagellate symbioses are fundamental components of coral reefs and other tropical ecosystems. The biochemical and molecular mechanisms underlying such symbiotic relationships remain poorly understood, although important efforts have been carried out to describe transcription profiles in several cnidarian-dinoflagellate systems (Weis & Levine, 1996; Richier et al., 2008; DeSalvo et al., 2010). Due to the difficulty of establishing appropriate models for the study of coral-dinoflagellate symbiosis, new emerging models such as *Aiptasia pulchella*, *Anemonia viridis* anemonae, and the jellyfish *Cassiopea xamachana*, have been used as model systems for various biochemical, molecular and transcriptomics approaches (Kuo et al., 2004; Markell & Wood-Charlson, 2010; Moya et al., 2012). The jellyfish *C. xamachana* offers various advantages for such studies since it can be propagated both sexually and asexually. The sexual cycle occurs when the male and female gametes produce a planula larva, which can settle and metamorphose to a polyp or scyphistoma (Colley & Trench, 1983). This scyphistoma can then acquire symbionts and differentiate to an ephyra, which will subsequently become an adult jellyfish (Fig. 1). If the scyphistomae do not acquire the symbiont, they can bud out new larvae, which can settle again and form new scyphistomae to perpetuate the cycle (Fig. 1; Colley & Trench, 1983). This physiological process represents an advantage to study the metamorphosis of the jellyfish under controlled laboratory conditions. However, in our hands, we have obtained inconsistent results with the induction of metamorphosis in *C. xamachana* with the infecting symbiont. Furthermore, we have consistently observed symbionts within our asexual scyphistomae cultures, which stay perpetuating the cycle without strobilation nor progression to the expected metamorphosis. Since we are interested in studying signal-transduction processes that occur during the metamorphic process, we required a reproducible and consistent way of inducing the metamorphosis in *C. xamachana* scyphistomae.

Several compounds have been reported for chemical induction of metamorphosis in jellyfish, mostly *Aurelia aurita*, which does not undergo symbiosis with *Symbiodinium*. These include indomethacin (Kuniyoshi et al., 2012), H_2O_2 (Berking et al., 2005), thyroxine and iodine (Spangenberg, 1967; 1974), and the indole compounds retinol, 5-methoxy-2-methyl indole acetic acid, 5 methoxyindole-2 carboxylic acid, 2-methylindole, and 5 methoxy-2-methylindole (Fuchs et al., 2014). One report documenting the use of the iodine-containing compound lugol as inducer of metamorphosis in *Cassiopea* spp. jellyfish exists (Pierce, 2005). In that study, 100% of strobilation was shown to occur after a week of exposure to 0.06 ppm. However, the induction of strobilation in the scyphistomae of this jellyfish with a single defined compound has not been documented.

In this work, we were able to consistently and reproducibly induce metamorphosis in *C. xamachana* scyphistomae by applying a single dose within a range of 0.5-50 µM indomethacin at 25 ± 2 °C and 200 µmole quanta m^{-2} s^{-1} under 12 h light/dark photoperiod cycles. These results place indomethacin as a tool for biochemical and/or molecular studies through a controlled metamorphic process in *C. xamachana* scyphistomae.

Materials and Methods

Animal rearing

Cassiopea xamachana scyphistomae were a kind gift of the Regional Center of Fisheries Research (Centro Regional de Investigaciones Pesqueras) in Puerto Morelos, Quintana Roo, México. The animals were reared in Petri plates containing filtered seawater and kept at 25 ± 2

ºC under darkness and only exposed to artificial laboratory light when fed. They were fed a diet of live *Artemia salina* nauplii every two days and cleaned from debris after feeding.

Chemicals

Thyroxine, KI, NaI, lugol's iodine (potassium tiiodide), indomethacin, retinol and dimethylsulfoxide (DMSO) were from Sigma. H_2O_2 was purchased from the local pharmacy.

Experimental treatments

The animals were stopped from feeding two days prior to exposure to the chemicals. The treatments were applied under the laboratory artificial ambient light and when started, the scyphistomae were placed under a 12 h light/dark cycle at an illumination of 70 µmoles quanta m^{-2} s^{-1}. Five scyphistomae were placed into individual wells of a microtiter plate and triplicate wells were used for each experimental treatment. The treatments were as follows: thyroxine at 0.1, 1, 5, 10, 20, 50 and 100 µM; retinol at 0.5, 1 and 5 µM; 1, 10 and 100 nM H_2O_2; 100 µM glucose; 100 µM glycine; 50, 100 and 300 µM L-tyrosine; 50, 100 and 300 µM NaI; 100 µM KI; 0.01% (v/v) glycerol; and lugol at 263 µL/L (equivalent to 130 mg/mL of iodine). Indomethacin was tested at 0.5, 1, 5, 10, 25, 50, 100, 200 and 500 µM. Controls consisting of filtered seawater with or without DMSO (as indomethacin was dissolved in DMSO) were also used.

Microscopy

Induction of metamorphosis to strobilation was monitored visually under a Leica MZ125 (Leica Microsystems) stereomicroscope. In order to monitor for the presence of symbionts inside the various stages of the animals, observations was carried out under a Zeiss Axioskop epifluorescence microscope with a rhodamine filter. Larvae, scyphistomae or strobile were

144 previously anesthetized by incubating them for 10 min with 10% $MgCl_2$ in filtered seawater at
145 25 ± 2 ºC, and then placed on the microscope slides for the observations.

146 **Statistical analysis**

147 Data were statistically analyzed using the R project software (www.r-project.org) with a Nested
148 ANOVA (days within different concentrations of indomethacin) and a Student-Newman-Kleus
149 post hoc analysis.

150 **Results**

151 **Symbionts are present at various stages of non-strobilating *C. xamachana***

152 In our hands, asexually reared *C. xamachana* at different physiological stages (maintained in the
153 dark and placed at ambient light only for feeding), consistently showed the presence of
154 symbionts. Larvae were observed to contain the endosymbionts observed as dark spots under
155 light microscopy (Fig. 2a, arrows). The same spots showed the characteristic chlorophyll
156 autofluorescence under fluorescence microscopy (Fig. 2d, arrows). Similarly, endosymbionts
157 were also consistently detected in tentacles at the scyphistoma stage under both light (Fig. 2b)
158 and fluorescence (Fig. 2e) microscopy. Even though endosymbionts had been clearly acquired in
159 these two physiological stages, infected scyphistomae did not strobilate and/or differentiate to
160 ephyrae. Comparatively, a strobilating scyphistoma also contained a significant load of
161 endosymbionts (Figs. 2c and f). Thus, in our hands, we obtained inconsistent results with the
162 induction of strobilation and metamorphosis in *C. xamachana* with the symbiont. Therefore, we
163 sought alternative methods to induce a reproducible and synchronous scyphistomae strobilation
164 and subsequent metamorphosis.

Indomethacin reproducibly induces strobilation

After testing several chemicals in an attempt to induce strobilation in *C. xamachana* scyphistomae (see below), we found a consistent induction with indomethacin whereas no induction was observed when plain seawater or seawater with the vehicle DMSO were used as negative controls (Fig. 3). We tested a range of 0.5 to 500 μM indomethacin concentrations to induce strobilation. A nested ANOVA analysis indicated significant differences between concentrations (DF=6, F=73.022, $p=2.2E^{-16}$) and days within each concentration (DF=21, F=12.889, $p=1.57E^{-14}$). A Student-Newman-Kleus post hoc analysis grouped days within each concentration ($p<0.01$) as denoted by letters. Strobilation of some scyphistomae began on the 5th d when the indomethacin concentration was at least 5 μM (Fig. 4, white bar) but it was not uniform and only 50% strobilation was observed at 50 μM concentration at this time (Fig. 4, white bar). Scyphistomae began to strobilate with a maximum difference of only 24 h, and all the indomethacin concentration treatments promoted strobilation after 6 d (Fig. 4, light gray bar). The indomethacin concentrations of 0.5-5 μM were directly proportional to the % strobilation up to the 6th day; however, strobilation was uniform after the 7th day. Strobilation seemed to induce a spontaneous synchrony of all the strobila since release of ephyrae occurred in all of them at 7 d independent of their time of strobilation. Thus, the optimum indomethacin concentration for a maximum strobilation induction in a shorter period of time (6 d) was 50 μM. Indomethacin at 50 μM also induced strobilation in the dark but the maximum was achieved at 10 d (not shown), indicating that the lack of photoperiod affects the process negatively. In addition, a lower temperature of 22 °C also delayed the strobilation process to 10 d (not shown). These data suggest that this process could be further manipulated by temperature and illumination conditions to accelerate or delay metamorphosis. When higher concentrations of

100, 200 and 500 µM indomethacin were tested, they were lethal to the scyphistomae (not shown).

Only indomethacin yielded reproducible and consistent results

In addition to indomethacin, we tested glucose, glycine, glycerol, thyroxine, L-tyrosine, KI, NaI, potassium triiodide (lugol's iodine), H_2O_2, and retinol, as inducers of metamorphosis in *C. xamachana* scyphistomae under the same temperature and light conditions as indomethacin. We used thyroxine and some iodine chemicals because previous reports documented the use of this hormone and the iodine-based compound lugol to induce strobilation in jellyfish scyphistomae (Spangenberg, 1974; Pierce, 2005). Thyroxine yielded inconsistent results (not shown). In all cases, the concentrations were non-lethal but strobilation and ephyrae release were obtained only once with 10 µM thyroxine (not shown). On the other hand, 0.5, 1 and 5 µM retinol did not have any effect on the *C. xamachana* scyphistomae and the result was identical as the untreated or mock controls (Fig. 2). Similarly, glucose, glycine, glycerol, L-tyrosine, KI, NaI, lugol and H_2O_2 were used at a wide range of concentrations but yielded inconsistent or no induction as well (not shown).

Discussion

Indomethacin induction of metamorphosis occurred consistently and in a reproducible manner in *C. xamachana* scyphistomae. The induction was effective at a range of concentrations of 5 to 50 µM which was within the concentration range observed by Kuniyoshi et al. (2012) for *A. aurita* (2.5 to 20 µM). They reported that, in the case of *A. aurita* induction, the strobilation was dose-dependent, where metamorphosis was induced with the highest doses at 9 d and with the lowest ones at 14 d of treatment (Kuniyoshi et al., 2012). We obtained similar results in the sense that at

0.5-1 µM strobilation did not occur at 5 d, whereas it did happen at 5-50 µM. In addition, maximum % strobilation was achieved at 8 d with 10-50 µM, whereas a statistically significant lower % strobilation occurred with 1 µM indomethacin tratment (Fig. 4). Furthermore, strobilation was uniform after the 7th day in the 5-50 µM range. Conversely, thyroxine, which is the protocol inducer in *A. aurita*, yielded inconsistent results as it only caused strobilation occasionally, while all other chemicals had no effect.

We do not know through which biochemical mechanism is indomethacin capable of inducing strobilation in *C. xamachana* scyphistomae. Indomethacin is an inhibitor of the cyclooxygenase (COX) enzyme, and therefore of the prostaglandin (PG) biosynthesis; however, when other COX inhibitors (such as aspirin, ibuprofen, etc.) were used, they did not stimulate strobilation in *A. aurita.* Similarly, when the synthesis of arachidonic acid (which is the COX substrate in the prostaglandin biosynthesis pathway) was inhibited, strobilation did not occur (Kuniyoshi et al., 2012). Thus, the COX pathway of prostaglandin biosynthesis does not seem to be the mechanism by which indomethacin induces metamorphosis in these cnidarians. This is also consistent with conflicting results on indomethacin action in mammalian models, where it appears to be involved in multiple pathways. For example, indomethacin can inhibit the cyclooxygenase (COX) pathway for prostaglandin (PG) biosynthesis, which is in turn, synthesized from arachidonic acid (Smith et al., 2011). However, in some cases, indomethacin did not inhibit COX expression, suggesting that there is an alternative COX-independent indomethacin pathway (Tegeder et al., 2001). Recently, evidence at the proteomic level has suggested the involvement of the Wnt1 signaling pathway without COX activation upon indomethacin treatment in colon cancer cells (Cheng et al., 2013). This is consistent with the proposed role of the Wnt1 pathway in cnidarian developmental processes (Holstein, 2008).

Recently, a peptide hormone with structural similarity to indole strobilation inducer chemicals such as indomethacin has been described as the active molecule to induce strobilation in *A. aurita* (Fuchs et al., 2014). Thus, it is likely that indomethacin acts mimicking such peptide hormone action.

Conclusions

This work demonstrates that indomethacin can be used as a reliable chemical inducer of metamorphosis in *C. xamachana* scyphistomae in a consistent and reproducible manner and that this induction may be further manipulated with light and temperature. After the strobilation onset in all scyphistomae, they seem to spontaneously synchronize to produce ephyrae release on the same day. This reproducible chemical induction of strobilation provides a powerful tool for biological, biochemical and molecular analyses of the metamorphic process under controlled conditions.

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304 **Figure Legends**

305 Figure 1. Microscopic analysis of *Symbiodinium* presence on three physiological stages of
306 *Cassiopea xamachana*. Endosymbiotic *Symbiodinium* cells were observed by their contrast
307 against the tissues by light microscopy (a-c), or by their chlorophyll autofluorescence (d-f).
308 Symbionts can be observed as dark or as fluorescent red dots, respectively, in a larval bud (a, d),
309 scyphistoma tentacles (b, e) and strobile (c, f). The arrows clearly show the symbionts as some
310 dark dots (a) corresponding to the same fluorescent ones (d) in a larval bud.

311 Figure 2. Life cycle of *Cassiopea xamachana*. The cycle starts with sexual reproduction (1, solid
312 lines), when adult jellyfish release their gametes into the water column. There, sperm-fertilized
313 eggs become free-living larval ciliates. Once the swimming larvae identifies a suitable substrate,
314 it settles and develops into a scyphistomae. The final stage is thought to ensue once
315 *Symbiodinium* has been acquired by the scyphistomae, triggering metamorphosis, strobilation
316 and ephyrae formation. The ephyrae are released into the water column creating a free-living
317 jellyfish. In the asexual component (2, dashed lines), the scyphistoma develops a bud that is
318 released into the enviroment as larvae which . These settle and metamorphose to scyphistomae,

and the cycle perpetuates. In parallel, as the ephyra is released (3), it can regenerate into a newly formed scyphistoma (dotted lines) and enter the asexual part of the cycle.

Figure 3. Induction of strobilation with indomethacin. Indomethacin (50 μM) was used to induce strobilation on *C. xamachana* scyphistomae. All samples used for the strobilation induction contained symbionts, but only those treated with indomethacin (c) strobilated. Changes can be observed in the calyx of the scyphistomae at day 3, where they begin to show elongation. At day 4 the tentacles start to retract and at day 5 all the tentacles are absent and the strobile begins pulsating. On day 6 and 7, the ephyra matures and on day 8 it is released into the environment. In contrast to the Indomethacin treatment, the seawater (a) or DMSO (b) vehicle controls did not result in strobilation. The experiment was repeated over three times independently with the same results.

Figure 4. Induction of strobilation under increasing indomethacin concentrations. Indomethacin (0.5-50 μM) was used to induce strobilation on *C. xamachana* scyphistomae and percent strobilation recorded after 5 (white bars), 6 (light gray bars), 7 (dark gray bars), and 8 (black bars) d. Triplicate samples each containing five scyphistomae were used for each concentration (see Materials and methods). Experiments were reproducibly performed at least five times. Maximum strobilation within a shortest period of treatment was achieved with 50 μM indomethacin at 6 d. The bars show the average ± the standard deviation. Post hoc analysis is denoted by small letters at $p<0.01$.

Figure 1

Microscopic analysis of *Symbiodinium* presence on three physiological stages of *Cassiopea xamachana*.

Endosymbiotic *Symbiodinium* cells were observed by their contrast against the tissues by light microscopy (a-c), or by their chlorophyll autofluorescence (d-f). Symbionts can be observed as dark or as fluorescent red dots, respectively, in a larval bud (a, d), scyphistoma tentacles (b, e) and strobile (c, f). The arrows clearly show the symbionts as some dark dots (a) corresponding to the same fluorescent ones (d) in a larval bud.

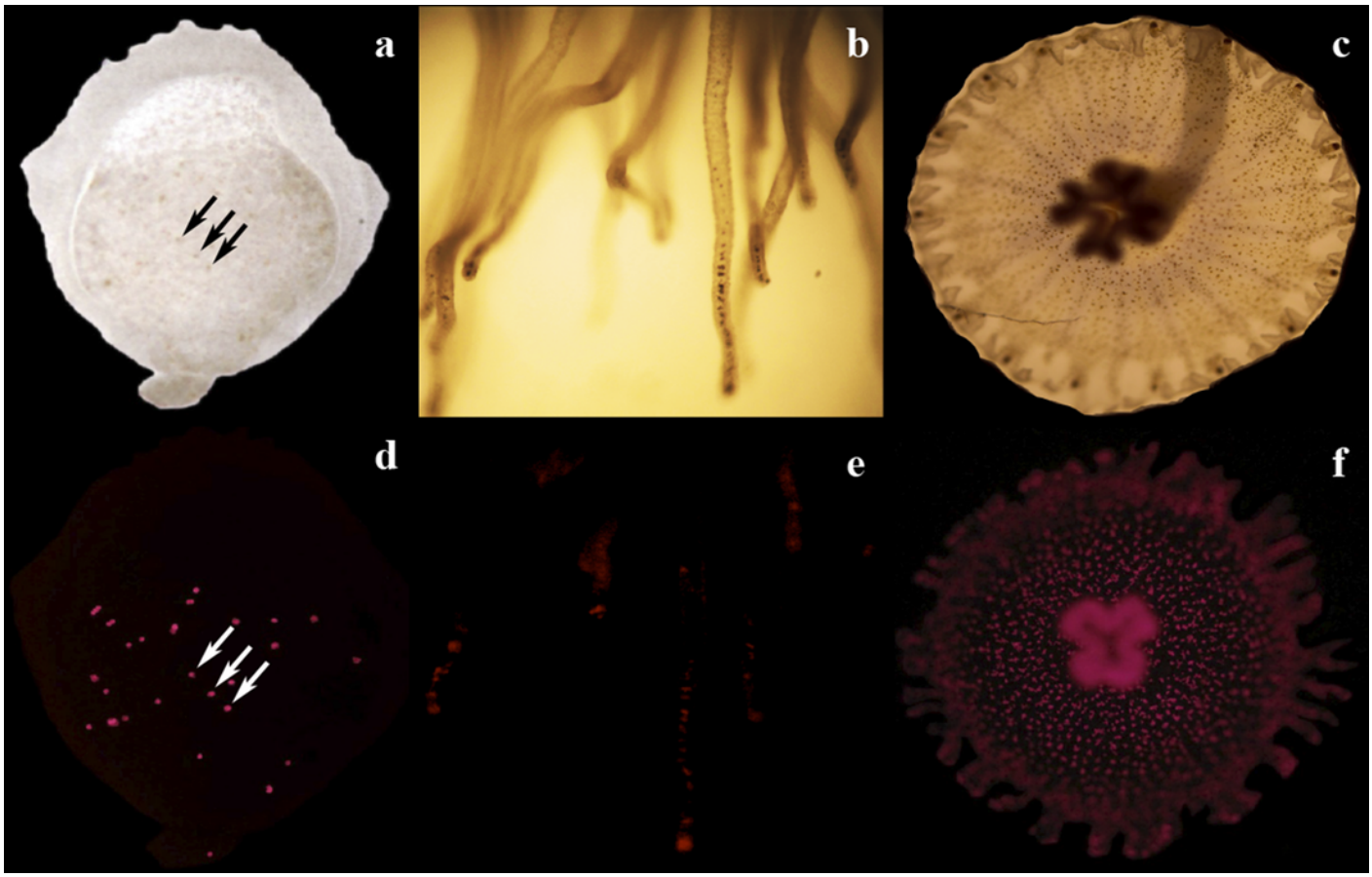

Figure 2

Life cycle of *Cassiopea xamachana*.

The cycle starts with sexual reproduction (1, solid lines), when adult jellyfish release their gametes into the water column. There, sperm-fertilized eggs become free-living larval ciliates. Once the swimming larvae identifies a suitable substrate, it settles and develops into a scyphistomae. The final stage is thought to ensue once *Symbiodinium* has been acquired by the scyphistomae, triggering metamorphosis, strobilation and ephyrae formation. The ephyrae are released into the water column creating a free-living jellyfish. In the asexual component (2, dashed lines), the scyphistoma develops a bud that is released into the enviroment as larvae which . These settle and metamorphose to scyphistomae, and the cycle perpetuates. In parallel, as the ephyra is released (3), it can regenerate into a newly formed scyphistoma (dotted lines) and enter the asexual part of the cycle.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

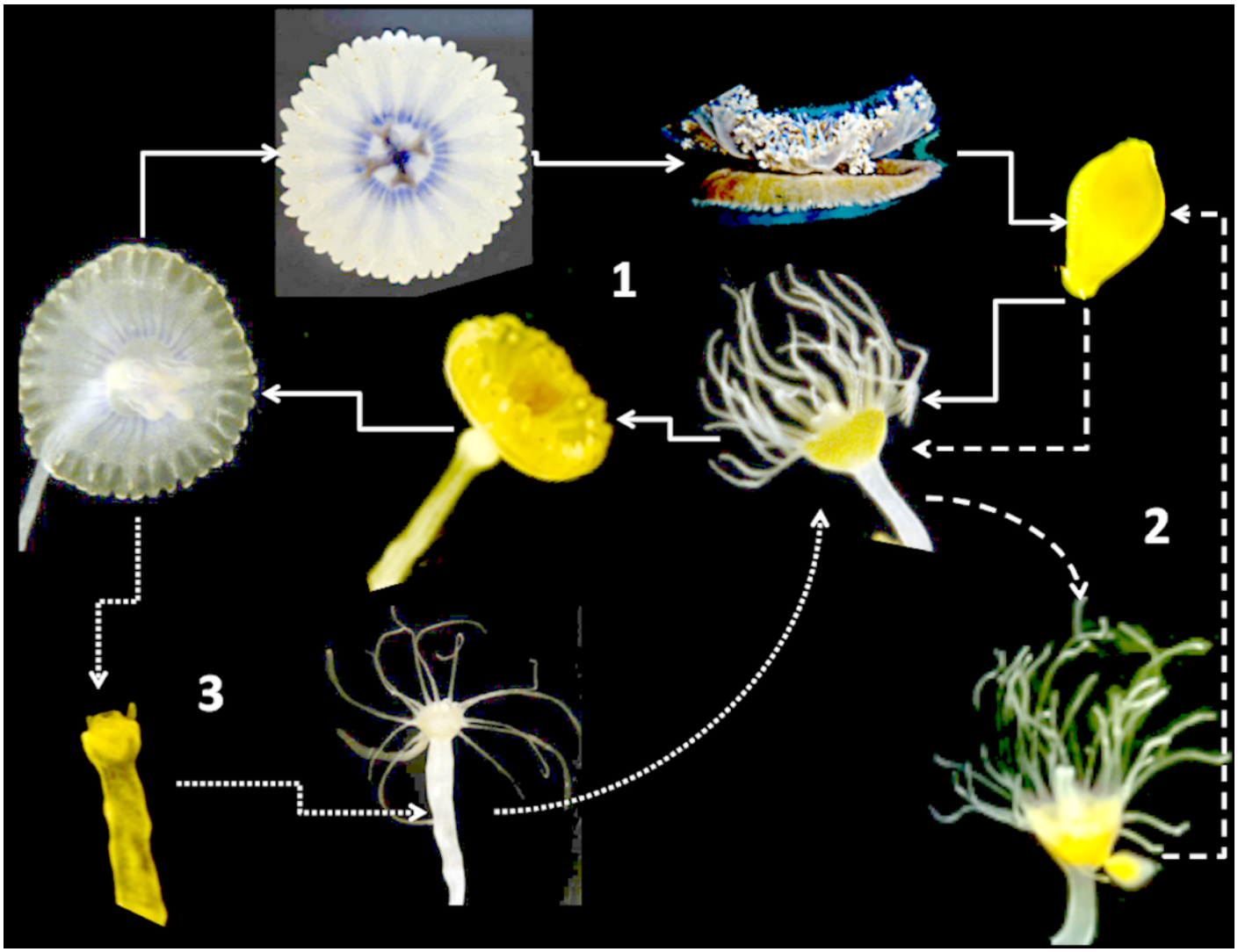
1
2
3

Figure 3

Induction of strobilation with indomethacin. Indomethacin (50 µM) was used to induce strobilation on *C. xamachana* scyphistomae.

All samples used for the strobilation induction contained symbionts, but only those treated with indomethacin (c) strobilated. Changes can be observed in the calyx of the scyphistomae at day 3, where they begin to show elongation. At day 4 the tentacles start to retract and at day 5 all the tentacles are absent and the strobile begins pulsating. On day 6 and 7, the ephyra matures and on day 8 it is released into the environment. In contrast to the Indomethacin treatment, the seawater (a) or DMSO (b) vehicle controls did not result in strobilation. The experiment was repeated over three times independently with the same results.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

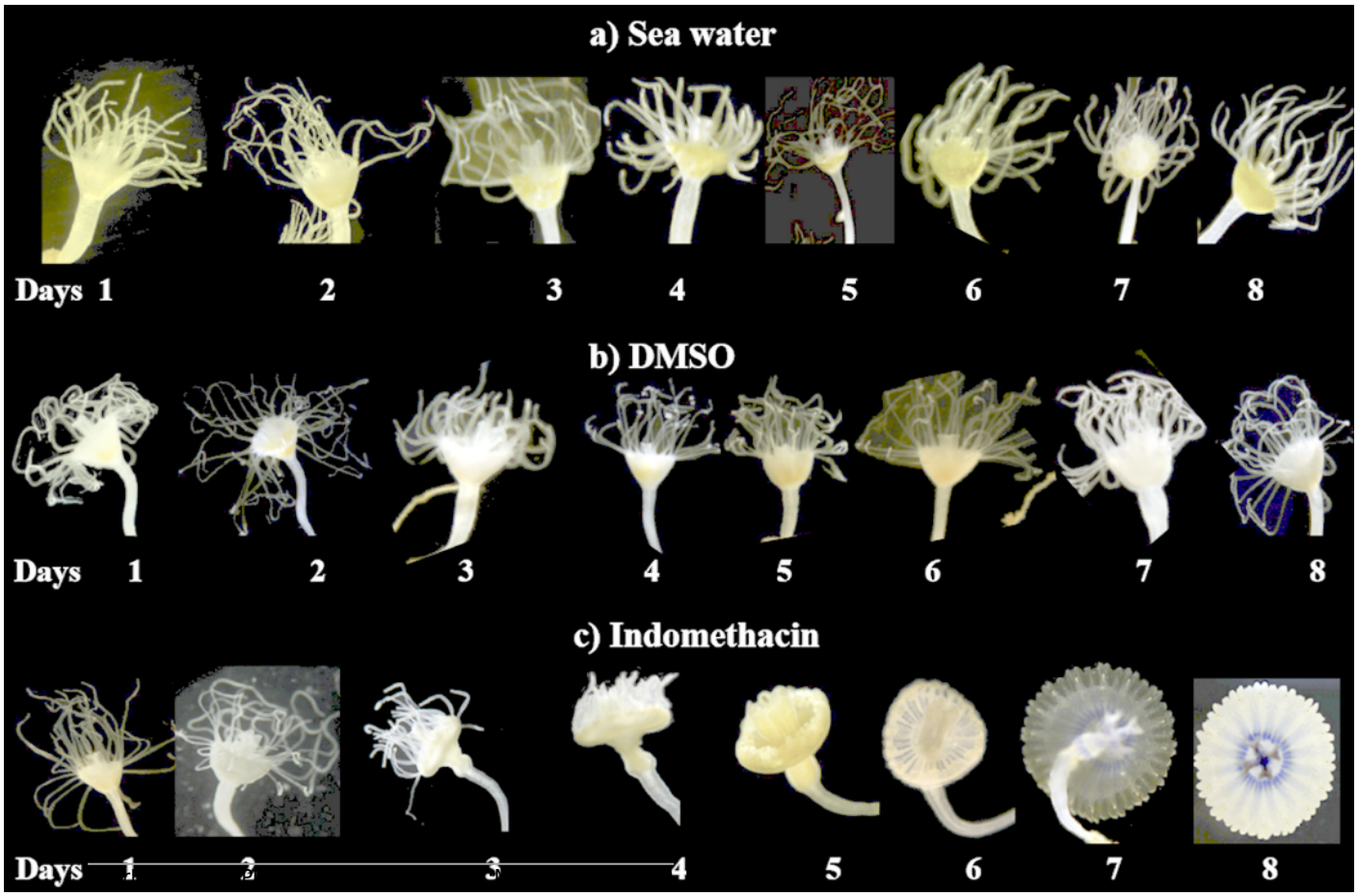

Figure 4

Induction of strobilation under increasing indomethacin concentrations.

Indomethacin (0.5-50 µM) was used to induce strobilation on *C. xamachana* scyphistomae and percent strobilation recorded after 5 (white bars), 6 (light gray bars), 7 (dark gray bars), and 8 (black bars) d. Triplicate samples each containing five scyphistomae were used for each concentration (see Materials and methods). Experiments were reproducibly performed at least five times. Maximum strobilation within a shortest period of treatment was achieved with 50 µM indomethacin at 6 d. The bars show the average ± the standard deviation. Post hoc analysis is denoted by small letters at $p<0.01$.