Trichoplax adhaerens reveals a network of nuclear receptors sensitive to 9-*cis*-retinoic acid at the base of metazoan evolution

Jan Philipp Novotný [1, 2] , **Ahmed Ali Chughtai** [1] , **Markéta Kostrouchová** [1, 3] , **Veronika Kostrouchová** [1] , **David Kostrouch** [1] , **Filip Kaššák** [4] , **Radek Kaňa** [5] , **Bernd Schierwater** [6, 7] , **Marta Kostrouchová** [1] , **Zdenek Kostrouch** Corresp. [1]

[1] Biocev, First Faculty of Medicine, Charles University, Vestec, Czech Republic

[2] Department of Medicine V., University of Heidelberg, Heidelberg, Germany

[3] Department of Pathology, Third Faculty of Medicine, Charles University, Prague, Czech Republic

[4] Biocev, Charles University, Vestec, Czech Republic

[5] Institute of Microbiology, Laboratory of Photosynthesis, Czech Academy of Sciences, Třeboň, Czech Republic

[6] Institute for Animal Ecology and Cell Biology, University of Veterinary Medicine, Hannover, Germany

[7] Department of Molecular, Cellular and Developmetnal Biology, Yale University, New Haven, CT, United States

Corresponding Author: Zdenek Kostrouch
Email address: Zdenek.kostrouch@lf1.cuni.cz

Trichoplax adhaerens, the only known species of Placozoa is likely to be closely related to an early metazoan that preceded branching of Cnidaria and Bilateria. This animal species is surprisingly well adapted to free life in the World Ocean inhabiting tidal costal zones of oceans and seas with warm to moderate temperatures and shallow waters. The genome of *T. adhaerens* (sp. Grell) includes four nuclear receptors, namely orthologue of RXR (NR2B), HNF4 (NR2A), COUP-TF (NR2F) and ERR (NR3B) that show a high degree of similarity with human orthologues. In the case of RXR, the sequence identity to human RXR alpha reaches 81% in the DNA binding domain and 70 % in the ligand binding domain. We show that *T. adhaerens* RXR (TaRXR) binds 9-*cis* retinoic acid (9-*cis*-RA) with high affinity, as well as high specificity and that exposure of *T. adhaerens* to 9-*cis*-RA regulates the expression of the putative *T. adhaerens* orthologue of vertebrate L-malate-$NADP^+$ oxidoreductase (EC 1.1.1.40) which in vertebrates is regulated by a heterodimer of RXR and thyroid hormone receptor. Treatment by 9-*cis*-RA alters the relative expression profile of *T. adhaerens* nuclear receptors, suggesting the existence of natural ligands. Keeping with this, algal food composition has a profound effect on *T. adhaerens* growth and appearance. We show that nanomolar concentrations of 9-*cis*-RA interfere with *T. adhaerens* growth response to specific algal food and causes growth arrest. Our results uncover an endocrine-like network of nuclear receptors sensitive to 9-*cis*-RA in *T. adhaerens* and support the existence of a ligand-sensitive network of nuclear receptors at the base of metazoan evolution.

Trichoplax adhaerens reveals a network of nuclear receptors sensitive to 9-*cis*-retinoic acid at the base of metazoan evolution.

Jan Philipp Novotný[1,2], Ahmed Ali Chughtai[1], Markéta Kostrouchová[1,3], Veronika Kostrouchová[1], David Kostrouch[1], Filip Kaššák[1], Radek Kaňa[4], Bernd Schierwater[5,6], Marta Kostrouchová[1], Zdeněk Kostrouch[1*]

1 Biocev, First Faculty of Medicine, Charles University, Vestec, Czech Republic

2 Department of Medicine V, University of Heidelberg, Heidelberg, Germany

3 Department of Pathology, Third Faculty of Medicine, Charles University, Ruská 87, 10000 Praha 10, Czech Republic

4 Laboratory of Photosynthesis, Centrum Algatech, Institute of Microbiology, Academy of Sciences Novohradska 237 - Opatovicky mlýn, CZ 379 01 Třeboň, Czech Republic.

5 Division of Ecology and Evolution, Stiftung Tierärztliche Hochschule, Hannover Germany

6 Department of Molecular, Cellular and Developmental Biology, Yale University New Haven, CT, USA

*Corresponding author:

Zdenek Kostrouch

Biocev, First Faculty of Medicine,

Charles University,

Průmyslová 595, 252 42 Vestec,

Czech Republic

email: zdenek.kostrouch@lf1.cuni.cz

ABSTRACT

Trichoplax adhaerens, the only known species of Placozoa is likely to be closely related to an early metazoan that preceded branching of Cnidaria and Bilateria. This animal species is surprisingly well adapted to free life in the World Ocean inhabiting tidal costal zones of oceans and seas with warm to moderate temperatures and shallow waters. The genome of *T. adhaerens* (sp. Grell) includes four nuclear receptors, namely orthologue of RXR (NR2B), HNF4 (NR2A), COUP-TF (NR2F) and ERR (NR3B) that show a high degree of similarity with human orthologues. In the case of RXR, the sequence identity to human RXR alpha reaches 81% in the DNA binding domain and 70 % in the ligand binding domain. We show that *T. adhaerens* RXR (TaRXR) binds 9-*cis* retinoic acid (9-*cis*-RA) with high affinity, as well as high specificity and that exposure of *T. adhaerens* to 9-*cis*-RA regulates the expression of the putative *T. adhaerens* orthologue of vertebrate L-malate-$NADP^+$ oxidoreductase (EC 1.1.1.40) which in vertebrates is regulated by a heterodimer of RXR and thyroid hormone receptor. Treatment by 9-*cis*-RA alters

the relative expression profile of *T. adhaerens* nuclear receptors, suggesting the existence of natural ligands. Keeping with this, algal food composition has a profound effect on *T. adhaerens* growth and appearance. We show that nanomolar concentrations of 9-*cis*-RA interfere with *T. adhaerens* growth response to specific algal food and causes growth arrest. Our results uncover an endocrine-like network of nuclear receptors sensitive to 9-*cis*-RA in *T. adhaerens* and support the existence of a ligand-sensitive network of nuclear receptors at the base of metazoan evolution.

INTRODUCTION

Life on Earth began 4.1 to 3.5 billion years ago (Bell et al. 2015) with the appearance of the first unicellular organisms that subsequently evolved, in part, to multicellular lifeforms forming the kingdom Metazoa that have specialized tissues for digestion, regulation of homeostasis, locomotion, perception, analysis of the environment and reproduction.

In contrast to unicellular organisms, metazoans are in need of regulatory mechanisms that provide the means of coordination between various tissues in a tight arrangement with cellular homeostasis. This coordination on the level of humoral signaling includes regulation by nuclear receptors (NRs), which respond to small, mostly hydrophobic molecules, including hormones produced by specific tissues, metabolites or even molecules present in the environment and transfer these signals to the nucleus, and thus leading to a dynamically changing but adaptive gene expression (Escriva et al. 2004).

NRs therefore play an important role in maintaining intra- and inter-cellular functions in multicellular organisms. Their overall structure is common in most nuclear receptors and consists

of an A/B (N-terminal) domain, the DNA binding domain (DBD), a hinge region, the ligand binding domain (LBD) and the C-terminal domain (Kumar & Thompson 1999; Robinson-Rechavi et al. 2003). The DBD and LBD of NRs exhibit an especially high degree of conservation and the changes that were acquired during evolution allow classification of the NR protein family into six subfamilies (Laudet 1997; Escriva et al. 1998). NRs that evolved within these subfamilies show functional connections that include specialization of regulatory functions in time or cell type restriction, fortification of ancestral functions or their specific inhibition by newly evolved NRs (Escriva et al. 2004; Kostrouchova & Kostrouch 2015).

With the overall structure maintained across metazoan species, nuclear receptors show significant heterogeneity regarding their quantity and function, many of which have not yet been explored in e.g. *Caenorhabditis elegans* with over 280 nuclear receptors (reviewed in (Kostrouchova & Kostrouch 2015)).

The evolutionary changes accumulated in diversified NRs allow functional subspecialization at the level of specific sequence binding within gene promoters (response elements), protein-protein interactions with functionally linked receptor interactors and adoption of new ligands as specific hormonal regulators. The evolution of hormonal ligands acquired by different species during evolution is well documented and indicates the potential of NRs to adopt new ligands as regulators (Escriva et al. 2000; Markov & Laudet 2011). Indeed, it is now thought that NRs evolved as environmental sensors that were able to sense a wide variety of compounds with low affinity and specificity, some of which later-on acquired higher affinity binding towards certain ligands that are products of metabolic pathways (Holzer et al. 2017). This can be exemplified by the high affinity and specificity binding of certain receptors, such as the mineralocorticoid or androgen receptors, while the family of PPARs shows a rather promiscuous

binding to a variety of different substances (Issemann & Green 1990). Keeping in mind the metabolic origin of NR ligands, it is not completely surprising to see different ligands binding to orthologues across species, such as Triac and T3 in the case of TR (Escriva 2008, Bridgham 2008), thus changing in the course of evolution and adapting to new environments.

This is accompanied by the essential questions, to what degree the plasticity of ligand selection is a fundamental property of NRs and what the origin of specific ligand binding by NRs might be. It has been suggested that the original NR, which is the ancestral NR possessing gene regulatory capacity, may have been an unliganded molecular regulator (Escriva et al. 1997). However, it is now believed that the ancestral NR is most closely related to the NR2 subfamily, as members of this family can be found in basal metazoans and are sensors of fatty acids (Holzer et al. 2017). More recently, it was proposed that the ligand binding and ligand-dependent regulatory potential of NRs is an inherent feature of the evolution of NRs (Bridgham et al. 2010). Due to their nature of fine tuning cellular responses in response to environmental changes without necessarily showing high affinity binding to a set of ligands, cross-species comparison of nuclear receptor networks might shed light on the details of the NR network function (Holzer et al. 2017).

A search for NRs that may be closely related to an ancient ancestor of the NR family led to the discovery of an RXR orthologue in Cnidaria (Kostrouch et al. 1998). Surprisingly, this receptor showed not only extremely high degree of sequence identity with vertebrate RXRs, far surpassing the degree of conservation observed in insects but also by its ability to bind the same ligand as vertebrate RXRs, *9-cis*-retinoic acid (*9-cis*-RA), with an affinity close to that reported for vertebrate RXRs. Similarly, as vertebrate RXRs, the jellyfish RXR showed a specific binding preference for *9-cis*-RA over all-*trans*-retinoic acid (AT-RA) and was able to heterodimerize with vertebrate thyroid hormone receptor alpha. Recent genome sequencing projects confirmed the

existence of highly conserved RXR across several metazoan species including insects (*Locusta migratoria* (Nowickyj et al. 2008), that are evolutionarily older than species with a more diversified RXR orthologue such as Usp found in *Drosophila* (reviewed in (Gutierrez-Mazariegos et al. 2014)).

To date, the nuclear receptor network has mainly been studied in complex organisms already in possession of an extensive endocrine network. Albeit dissection of nuclear receptor networks in these organisms can outline functions and associated regulatory cascades, basal tasks might be obscured by the gain of further, more specialized functions. Genome analysis of the basal metazoan *Trichoplax adhaerens* by whole genome sequencing revealed four highly conserved nuclear receptors, namely orthologues of HNF4 (NR2A), RXR (NR2B), ERR (NR3B) and COUP-TF (NR2F) (Baker 2008; Srivastava et al. 2008) and thus allows assessment of the most basal workings of nuclear receptors. Surprisingly, the degree of conservation of the predicted placozoan NRs with known vertebrate NRs is not only very high at the level of the predicted secondary structure, as can be expected for true NRs, but also at the level of the primary amino acid sequence. Especially the similarity of the placozoan RXR (TaRXR) to its vertebrate orthologues is high, as it is in the case of the cubomedusan RXR. *T. adhaerens,* which shows characteristics of a basal metazoan with only a few cell types (Smith et al. 2014) and a relatively simple 4 member NR complement, offers a unique model that may shed light on the evolution of gene regulation by NRs.

In this presented work, we attempted to study the placozoan RXR orthologue functionally. Our results show that *T. adhaerens* RXR binds 9-*cis*-RA with an affinity comparable to that of vertebrate and jellyfish RXRs and that *T. adhaerens* responds to nanomolar concentrations of 9-*cis*-RA with a transcriptional upregulation of the putative orthologue of a malic enzyme that is

regulated by a heterodimer formed by liganded thyroid hormone receptor and RXR in vertebrates. We also show that 9-*cis*-RA affects the relative expression of the four NRs present in *T. adhaerens* genome suggesting that these NRs may form a regulatory network capable of responding to possible ligands present in these animals or their environment. In line with this, growth, multiplication and appearance of *T. adhaerens* are strongly affected by food composition, especially by red pigment containing algae suggesting that specific food components or their metabolites may be ligands involved in the ancestral regulatory network of NRs. In support of this, 3.3 nM 9-*cis*-RA interferes with *T. adhaerens* growth response to the feeding by *Porphyridium cruentum* and causes balloon-like phenotypes and death of animals while animals fed by *Chlorella sp*. are partially protected against the treatment by 3.3 nM 9-*cis*-RA, do not develop balloon-like phenotypes but are also arrested in their growth and propagation indicating that 9-*cis*-RA interferes with *T. adhaerens* growth and development.

METHODS

Bioinformatics and cloning of RXR

The predicted RXR gene models on jgi (http://jgi.doe.gov/) (Nordberg et al. 2014) were screened for the characteristic molecular signature of the DNA binding domain (C-X2-C-Xl3-C-X2-C-X15-C-X5-C-X9-C-X2-C-X4-C-X4-M) (Kostrouch et al. 1995) and the appropriate predicted gene model (protein ID 53515) was selected for further use.

The alignment of different RXRs was performed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al. 2011) and adjusted/exported as an image file using Jalview (http://www.jalview.org). Protein domain characterization was performed

158 with SMART (Schultz et al. 1998; Letunic et al. 2015). Analysis of HNF4, ERR and COUP-TF
159 was done similarly. Phylogenetic analysis was performed on RXR ClustalO alignment using
160 PhyMLv3.1 (Guindon et al. 2010) implemented in SeaView v4.6.1 with a 100 bootstrap analysis
161 and SPR distance computation. The tree was then visualized using FigTree v1.4.3.

162 *T. adhaerens* total RNA was obtained from 50-100 pooled individual animals and extracted
163 using TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol supplied by
164 the manufacturer.

165 Subsequently, cDNA was prepared with random hexamers and SuperScript III
166 (Invitrogen™) according to the manufacturer's protocol.

167 Several RXR transcripts were then amplified by PCR with primers covering the starting sequence
168 ((GCGGATCC)ATGGAGGACAGATCGTTTAAAAAA), starting at 32 bp 5' of ATG
169 (TCTACCAATGTTTATCGCATCGGTTA) and starting at 97 bp 5' of ATG
170 (TTAAGGCTTAACTGATGATGTTGTGAATG) with a common reverse primer covering the
171 last 24 bp of the predicted gene sequence
172 ((CGGAATTC)TTAAGAACTGCCTGTTTCCAGCAT).

173 Each PCR product was then ligated into pCR®2.1-TOPO® or pCR®4-TOPO® vector
174 with the classic TA Cloning Kit and TOPO TA Cloning Kit (Invitrogen™), respectively. The
175 ligated products were then transformed using One Shot® TOP10 Chemically Competent *E. coli*
176 and cultured on LB Agar plates containing 100 μg/ml ampicillin. Plasmid DNA was extracted
177 from obtained colonies and screened for mutations by sequencing using vector specific M13
178 forward and reverse primers. Only non-mutated sequences were used in subsequent experiments.
179 The RXR fragments were then restricted and inserted into pGEX-2T vector system for bacterial
180 expression (Addgene, Cambridge, MA, USA). Proper insertion was verified by sequencing.

Protein expression

Bl21 pLysS bacteria were transformed with previously described RXR mRNA inserted into pGEX-2T vector. Stocks of transformed bacteria were stored in 8% glycerol according to the Novagen pET System Manual (11th edition) (https://www.google.cz/search?q=Novagen+pET+System+Manual+&ie=utf-8&oe=utf-8&client=firefox-b&gfe_rd=cr&ei=T9z1WMHJDsni8AfpmoGoCQ). For protein expression, bacteria were scraped from stock and incubated in Liquid Broth (LB) with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) overnight. The culture was then used to inoculate 100 ml of LB + antibiotics and grown to OD600 = 0.6-0.8 at 37 ºC, then induced with 100 µl 1M IPTG (isopropyl-D-thiogalactopyranoside) (Sigma-Aldrich, St. Louis, MO, USA) and moved to 25 ºC (RT) for 5 h. The culture was then spun at 9000 xg for 15 min and the supernatant discarded. The bacterial pellet was resuspended in 10 ml GST binding buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA + protease inhibitor (S8820 Sigma Fast, Sigma-Aldrich, St. Louis, MO, USA or cOmplete™, EDTA-free Protease Inhibitor Cocktail, Roche, Basel, Switzerland). Bacteria were then lysed by 6 x 20 s ultrasonication on ice (50 watts, 30kHz, highest setting – 100%) (Ultrasonic Processor UP50H, (Hielscher Ultrasonics GmbH, Teltow, Germany) and subsequently incubated with 15-20 mg glutathione agarose beads (Sigma-Aldrich®) prepared according to manufacturer's instructions. Incubation took place at 4 ºC for about 10 h after which the beads were washed according to instructions, resuspended in regeneration buffer (50mM Tris-HCl pH7.4, 1mM EDTA, 120 mM KCl, 5 mM DTT, 8% glycerol (v/v)) or 50mM TRIS–HCl pH 7.4 + 9% (v/v) glycerol for subsequent thrombin (bovine plasma, Sigma-Aldrich®) cleavage, if performed, and then adjusted for regeneration buffer conditions. GST-TaRXR was eluted from glutathione agarose

beads using 10 mM reduced glutathione (Sigma-Aldrich, StLouis, Mo, USA) in 50 mM Tris-HCl buffer pH 8.0. The size of the GST-TaRXR fusion protein was checked by polyacrylamide gel electrophoresis. Thrombin cleavage was performed at RT for 4 h and the quality of the purified protein was assessed by polyacrylamide gel electrophoresis.

Radioactive *9-cis RA* binding assay

Radioactive ^{3}H-labelled 9-*cis*-*RA* and ^{3}H-labelled AT-RA were purchased from PerkinElmer (Waltham, MA, USA). Binding was performed in 100 µl binding buffer (50mM Tris-HCl pH7.4, 1mM EDTA, 120 mM KCl, 5 mM DTT, 8% glycerol (v/v), 0.3% to 0.5% (w/v) CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, Sigma-Aldrich) for 2 h on wet ice in a dark environment. The protein used for binding was either GST-RXR fusion protein on beads with about 375 ng/assay and thrombin-cleaved RXR. For estimation of specific binding, 200x excess of either 9-*cis*-RA or AT-RA (Sigma-Aldrich) was used. In case of GST-RXR fusion protein, 50 µl of the supernatant was removed after 30 s at 1300 g and washed 3x with 1000 µl wash buffer (50 mM Tris-HCl pH7.4, 1 mM EDTA, 120 mM KCl, 5 mM DTT, 8% (v/v) glycerol, 0.5% (w/v) CHAPS) removing 900 µl after each wash. For cleaved RXR protein 10 µl hydroxyapatite slurry (AG-1 XB Resin, Bio-Rad, Hercules, CA, USA) suspended in binding buffer (12.7 mg/100µl) were added to the assay and mixed twice, collecting the apatite slurry by centrifugation (15 s at 600 g). 95 µl of the supernatant was removed and the slurry washed twice with 1 ml of wash buffer, removing 900 µl after each wash. Work with retinoids was done under indirect illumination with a 60W, 120V yellow light bulb (BugLite, General Electric Co, Nela Parc, Cleveland Oh, USA) as described (Cahnmann 1995). The radioactivity of the GST-fusion protein and cleaved protein was measured using Packard Tri-Carb 1600TR Liquid Scintillation

Analyzer (Packard, A Canberra Company, Canberra Industries, Meriden, CT, USA) and Ultima Gold Scintillation Fluid (PerkinElmer, Waltham, MA, USA). The fraction of bound ^{3}H-labelled 9-cis-RA and ^{3}H-labelled all-*trans*-RA was determined as a ratio of the bound radioactivity of precipitated GST-TaRXR / total radioactivity used at the particular condition (determined as the sum of bound radioactivity and the total radioactivity of collected wash fluids) in the absence of non-radioactive competitors or 200 fold excess of 9-cis-RA and all-trans-RA in the case of ^{3}H-labelled 9-*cis*-RA and 40 fold excess of non-radioactive competitors in the case of ^{3}H-labelled all-*trans*-RA (to compensate for the higher affinity of 9-*cis*-RA compared to all-*trans*-RA in binding to TaRXR).

Culture of *T. adhaerens* and algae

Trichoplax adhaerens was cultured in Petri dishes containing filtered artificial seawater (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) with a salinity of approx. 38-40 ppt. *Rhodomonas salina* (strain CCAP 978/27), *Chlorella sp*., *Porphyridium cruentum* (UTEX B637) and other non-classified algae, as well as aquarium milieu established in the laboratory by mixing salt water obtained from a local aquarium shop were used to maintain the stock. The cultures were kept at approx. 23°C and an automated illumination for 12 h/day was used with a conventional light bulb on a daylight background from late spring to mid-summer in the laboratory located at 50.07031N, 14.42934E with laboratory windows oriented eastward. The natural illumination included almost direct morning light from 8 AM to 10.30 AM, indirect sunlight for most of the daytime and sunlight reflected from a building across the street from 1 PM to 6 PM. Algae were maintained as described (Kana et al. 2012; Kana et al. 2014). The experiments were performed predominantly during sunny weather.

250

251 Treatment of *T. adhaerens* with retinoic acids

252 Incubation of the animals was done overnight in the absence of light. Each batch within an
253 experiment was derived from similar cultures and fed with similar amounts and composition of
254 algae. All experiments were started in a dark room with indirect yellow light illumination
255 (similarly as in the case of the ligand binding studies) and further incubations were done in the
256 dark for 24 hours. In experiments aimed at the visualization of 9-*cis*-RA effect on *T. adhaerens*
257 response to feeding conditions, parallel cultures were set and fed with *P. cruentum.* Large animals
258 of approximately the same size were individually transferred to new control and experimental
259 cultures and fed with *P. cruentum* algal cells. After 6 hours of incubation under natural indirect
260 illumination, all animals in both control and experimental cultures were photographed (max.
261 magnification on Olympus SZX7 with Olympus E-410 camera) and the final volume of cultures
262 was adjusted to 50 ml (determined by the weight of cultures in 110 mm glass Petri dishes). Next,
263 the room was darkened and further manipulations were done under indirect illumination with
264 yellow light. Five µl of vehicle (1% DMSO in ethanol) or vehicle containing 9-*cis*-RA was added
265 into 50 ml of final volume to the final concentration of 9-*cis*-RA 3.3 nM. Similarly, parallel sub-
266 cultures were prepared from slowly growing cultures fed by microorganisms covering glass slides
267 in an equilibrated 25 l laboratory aquarium and fed by *Chlorella sp*. Cultures were incubated in
268 the dark for 24 h and all animals were counted under microscope and photographed again. The
269 cultures were then left under natural illumination and cultured for an additional two or three weeks.
270 Animals fed by *P. cruentum* were measured again at 72, 90 and 450 h and those fed by *Chlorella*
271 *sp*. at 72, 90 and 378 h.

272 Quantitative PCR

Droplet digital PCR was performed on a QX100 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA). For this, *T. adhaerens* was cultured according to culture conditions described and 4-10 animals removed per 100µl TRIZol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was measured by a UV spectrophotometer and used as a reference for normalization.

Reverse transcription was performed with SuperScript III Reverse Transcriptase (ThermoFisher, Waltham, MA, USA) according to manufacturer's instructions. The cDNA (corresponding to 100-500 ng of RNA) was then mixed with ddPCR Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions and analyzed. PCR primers were designed using the UPL online ProbeFinder (Roche) software and were as follows:

TaRXR – left:tctgcaagttggtatgaagca, right: agttggtgtgctattctttacgc

TaHNF4 (ref|XM_002115774.1|):

left: ggaatgatttgattttacctcgac, right: tacgacaagcgatacgagca

TaCOUP-TF (ref|XM_002109770.1):

left: attttgaatgctgcccaatg, right: ttactggttgtggagtatggaaac

TaSoxB1 (ref|XM_002111308.1|):

left: tgtcagatgcggataaacga, right: ggatgttccttcatgtgtaatgc

TaTrox-2 (ref|XM_002118165.1|):

left: gcctatagtcgatcctgccata, right: ttggtgatgatggttgtcca

TaPaxB1 (gb|DQ022561.1):

left: tcaaacgggttctgttagcc, right: ggtgttgccaccttaggc

TaERR (nuclear receptor 3, gb|KC261632.1):

left: ttacgcatgtgatatggttatgg, right: agcgtgcctatttatttcgtct

296

297 Results were subsequently analyzed using the Bio-Rad ddPCR software. Manual
298 correction of the cut off was performed when automated analysis was not possible. To visualize
299 changes in nuclear receptor expression in the absence of a reliable housekeeping gene as a
300 reference, we considered the absolute quantity of each nuclear receptor as a percentage of the
301 overall nuclear receptor expression and subsequently visualized the change of receptor expression
302 by subtraction of the percentage of the control experiment. Absolute copy numbers of the proposed
303 malic enzyme orthologue in *T. adhaerens* have been normalized to overall RNA quantity for
304 expressional analysis.

305 Experiments with quantification by qRT-PCR were performed on a Roche LightCycler II
306 with OneTaq polymerase and the same probes as for ddPCR.

307 For the estimation of the relative expression of NRs in small (< 0.5 mm) versus big
308 animals (>1 mm), 20 to 30 animals from the same culture were used for each paired
309 experiment.

310

311 **Identification of *T. adhaerens* orthologue of L-malate-NADP$^+$ oxidoreductase (EC 1.1.1.40)**

312 P48163 (MAOX_HUMAN) protein sequence was used as the query sequence and
313 searched against *T. adhaerens* database with BLASTP on http://blast.ncbi.nlm.nih.gov/Blast.cgi
314 using standard algorithm parameters. The best hit was a hypothetical protein
315 TRIADDRAFT_50795 with a sequence identity of 57% and a query coverage of 93% and was
316 assumed to be *T. adhaerens* closest orthologue of vertebrate L-malate-NADP$^+$ oxidoreductase.

317

318 ## Microscopy and image analysis

Observation of *T. adhaerens* was done with an Olympus SZX10 microscope equipped with DF Plan 2x objective and Olympus DP 73 camera operated by CellSens Dimension computer program (kindly provided by Olympus, Prague, Czech Republic) or Olympus CKX41 or SZX7 with Olympus E-410 camera and QuickPhoto Micro 3.1 program.

Circularity was calculated by establishing the area (A) and perimeter (p) of *T. adhaerens* using ImageJ (https://imagej.nih.gov/ij/) and then calculated with the isoperimetric quotient $Q = \frac{4\pi A}{p^2}$, (A – Area, p – perimeter). GraphPad Prism 5 (or higher) was used for graphical representation and calculations of the confidence intervals with p = 0.05.

RESULTS

T. adhaerens retinoid X receptor shows high cross-species sequence identity

By using the ab initio model of the JGI *Trichoplax* database as a reference, we screened the *Trichoplax* JGI database for RXR orthologues with a complete DBD and LBD sequence and were able to obtain, as well as verify a full length RXR transcript previously not annotated as the 'best model'. Blastp analysis showed a high sequence similarity to human, as well as mouse RXR with 66% overall sequence identity to human RXR alpha.

SMART analysis of the proposed TaRXR sequence showed a zinc finger DNA binding domain (amino acid residues 16-87) and a ligand binding domain (amino acid residues 155-342) with E values $<10^{-40}$. Blast analysis of the zinc finger DNA binding and ligand binding domains revealed a sequence identity of 81% and 70% to human RXR alpha, respectively. Both domains contained the predicted molecular pattern characteristic for each domain. The heptad repeat

LLLRLPAL proposed for dimerization activity (Forman & Samuels 1990b; Forman & Samuels 1990a; Kiefer 2006) as well as the LBD signature for 9-*cis*-RA binding Q-x(33)-L-x(3)-F-x(2)-R-x(9)-L-x(44)-R-x(63)-H (Egea et al. 2000) were present (**Fig. 1**). From 11 amino acid residues shown to be critical for 9-*cis*-RA binding (A271, A272, Q 275, L 309, F 313, R 316, L 326, A 327, R 371, C 432, H 435), 9 are conserved, while the remaining two amino acids are substituted [A327S, and C432T (C432A in *Tripedalia cystophora*)]. Due to the high sequence identity, we propose a 9-*cis*-retinoic acid binding capability of the hypothesized TaRXR sequence, as well as DNA binding capability. Phylogenetic analysis using PhyML algorithm indicates that TaRXR is likely to precede branching of RXRs in cubomedusae and scyphomedusae and clusters with RXRs in bilateria **(Supplementary Fig S1).**

The remaining three NRs identified in the *T. adhaerens* genome show also very high overall sequence identity with vertebrate orthologues. Alignments of *T. adhaerens* HNF4, COUP-TF and ERR with orthologues from selected species can be found in **Supplementary File S2**.

TaRXR shows preferential binding affinity to *9-cis* retinoic acid over all-*trans*-retinoic acid

In order to analyze the binding properties of TaRXR, we expressed TaRXR as a GST-fusion protein (GST-TaRXR) in bacteria which was then purified as a GST-fusion protein and used directly for binding studies or cleaved by thrombin and eluted as TaRXR. The binding of ^{3}H-labelled 9-*cis*-RA or ^{3}H-labelled all-*trans*-RA was determined by measuring total bound radioactivity and the radioactivity displaceable by 200 fold excess of nonradioactive competitors. Consistent with the high conservation of the LBD, the experiments showed that TaRXR prepared

as thrombin cleaved TaRXR or GST-TaRXR binds 9-*cis*-RA with high affinity and specificity (**Fig. 2 A and B**). The 9-*cis*-RA binding assay showed high affinity binding to GST-TaRXR with a saturation plateau from 5 nM to 10 nM (**Fig. 2 C**). In contrast, all-*trans*-retinoic acid did not show high affinity binding to TaRXR of GST-TaRXR.

9-*cis*-retinoic acid induces malic enzyme gene expression at nanomolar concentrations

Next, we searched whether 9-*cis*-RA has observable biological effects on *T. adharens* at nanomolar concentrations. We hypothesized that TaRXR is likely to be involved in the regulation of metabolic events. In vertebrates, RXR is a dimerization partner of TR and together, these two NRs are regulating a wide range of metabolic pathways. We, therefore, searched for an orthologue of vertebrate L-malate-$NADP^+$ oxidoreductase (EC 1.1.1.40) in *T. adhaerens* genome since this enzyme is an established reporter of the state of thyroid hormone dependent regulation (see Discussion).

The sequence of the *T. adhaerens* likely orthologue of vertebrate L-malate-$NADP^+$ oxidoreductase was retrieved from the *Trichoplax* genomic database together with its presumed promoter based on the predicted sequence (**Supplementary File S3).**

Droplet digital PCR showed an increased transcription of the predicted L-malate-$NADP^+$ oxidoreductase gene after incubation of *T. adhaerens* with 9-*cis*-RA, but not with all-*trans*-RA **(Fig. 3)**. In repeated experiments, we observed that the level of induction was higher at 9-*cis*-RA concentrations in the range of 1 to 10 nM, than above 10 nM. We also noticed that the level of the induction slightly varied based on the actual *T. adhaerens* cultures and the algal food composition of the *T. adhaerens* cultures.

388

389

390 **Changes in the culture environment alter the expression pattern of the nuclear**
391 **receptor complement in *T. adhaerens***

392 From the experience we gained by culturing *T. adhaerens*, as well as from the previous
393 experiments we knew that the culture conditions could dramatically influence phenotype. Having
394 the possible developmental functions of the ancestral NRs in mind, we raised the question whether
395 the expression patterns of the NRs reflect changes in phenotype.

396 Firstly, we assayed the relative expression of RXR against all three other NRs in small
397 versus big animals (<0.5 mm or >1mm). The relative proportion of the RXR expression compared
398 to the remaining NRs was found to be higher in big animals (33%) than in small animals (24%).
399 The treatment by 3.3 nM 9-*cis*-RA led to a dramatic increase of the relative expression of RXR in
400 comparison to the rest of the NR complement (51%), indicating that phenotypic changes are
401 connected with differential expression of NRs and that 9-*cis*-RA affects the expression of RXR.

402 In order to see the effect of 9-*cis*-RA on all NRs, we sampled and extracted RNA from
403 cultures containing the same number of big and small animals treated with different concentrations
404 of 9-*cis*-RA. The experimental cultures were started from the same original cultures and during
405 incubation were fed with *Chlorella sp.* only since this algal food showed to have the least effect
406 on *T. adhaerens* cultures. All four *T. adhaerens* NRs were quantified by either qRT-PCR or
407 ddPCR.

408 Analysis of NR expression pattern in animals incubated with different concentrations of 9-
409 *cis*-RA, revealed a relative increase in RXR expression at low nanomolar concentrations (<10 nM)
410 in repeated experiments. In contrast, further increase of 9-*cis*-RA resulted in smaller changes

411 compared to the expression pattern of NRs in control animals or even reverted the values observed
412 in low nanomolar conditions (**Fig. 4**).
413
414

415 Food composition dramatically changes the phenotype and the reproduction
416 rate of *T. adhaerens*

417 *T. adhaerens* retrieved from laboratory aquariums used for the stock cultures were
418 relatively similar in appearance and included small round animals containing approximately 50
419 cells and grew to animals with an approximate diameter of 0.2 mm and rarely were bigger. Their
420 rate of multiplication when transferred to Petri dishes was doubling in one month or even one
421 week, depending on whether the glass was covered by microbial and algal films established during
422 culturing in aquariums. We attempted to use several defined algae as artificial food. They included
423 *Pyrrenomonas helgolandii*, *Picocystis salinarium*, *Tetraselmis subcoriformis*, *Rhodomonas*
424 *salina*, *Phaeodactylum tricornutum*, *Porphyridium cruentum* and *Chlorella sp*. Individual
425 subcultures of *T. adhaerens* differed in the rate of propagation and appearance as well as colors
426 that were varying from greenish to brown and reddish taints depending on the food that was used
427 as singular species food or mixtures (**Fig. 5**). Also, contaminants from the original algal food,
428 which prevailed in some cultures, had an influence on *T. adhaerens* growth and behavior. In
429 controlled experiments, it became clear that some food components or their metabolites are
430 influencing growth and appearance of *T. adherens* more than food availability. When *T. adhaerens*
431 were fed with equal amounts of algal cells (although they differed in size and expected
432 digestibility), the addition of algae containing red pigments - Cryptophytes (*Pyrrenomonas*
433 *helgolandii* and *Rhodomonas salina*) or Rhodophyta (*Porphyridium cruentum*) - had a strong

positive effect on *T. adhaerens* growth (**Fig. 6**), especially in combination with the green algae *Chlorella sp.* (**Fig. 6**).

Furthermore, the addition of *Porphyridium cruentum* to *Chlorella sp.* resulted in a significant change in circularity, while feeding *T. adhaerens* with 'triple food' containing *Chlorella*, *Rhodomonas* and *Porphyridium* showed the most pronounced effect. Culturing *T. adhaerens* on either of the single foods showed similar isoperimetric values (**Fig. 7).**

9-*cis*-RA interferes with *T. adhaerens* growth response to specific algal food

In order to see the effect of 9-*cis*-RA on *T. adhaerens* in cultures, we exposed cultures kept in a naturally established laboratory microenvironment or fed by specific algal foods to 3, 5 and 10 nM 9-*cis*-RA. The slowly growing cultures kept in naturally established laboratory microenvironment did not show any gross morphological changes even in 10 nM 9-*cis*-RA during the period of one week. Contrary to that, cultures fed with mixed algal food incubated in the presence of 3 and 5 nM 9-*cis*-RA ceased propagation and most animals developed a balloon-like phenotype, and later darkened and decomposed.

For controlled experiments, cultures fed by *P. cruentum* or *Chlorella sp*. were incubated in the presence of vehicle (DMSO/ethanol) or vehicle containing 9-*cis*-RA at 3.3 nM final concentration. After 24 hours of incubation in the dark, control cultures fed by *P. cruentum* propagated normally while animals fed by *P. cruentum* and incubated with 9-*cis*-RA decreased their area and perimeter (**Fig. 8 A**). At 72 h of incubation, all animals fed by *P. cruentum* and treated by 3.3 nM 9-*cis*-RA developed the balloon-like phenotype and none of them survived 90 hours of exposure to 9-*cis*-RA (**Fig. 8 B and Supplementary Fig. S2**). Animals transferred from stationary cultures grown in a naturally established laboratory microenvironment and subsequently

fed by *Chlorella sp*. suffered initial loses at 24 h of incubation despite that their appearance seemed to be normal and well adopted to the new culture condition at time 0 (regarding feeding with algal food and immediately prior to addition of vehicle or 9-*cis*-RA to the culture and 6 h after the transfer from the parent cultures). Animals that survived the transfer and adopted to feeding by *Chlorella sp*., were not inhibited by exposure to 3.3 nM 9-*cis*-RA for 24 h (**Fig. 8 C**) and even showed a slight statistically not significant increase in their area and perimeter. Nevertheless, the isoperimetric values of animals incubated for 24 h with 9-cis-RA showed a significant increase indicating a decrease of growth or exhaustion of peripheral area, that is likely to contain stem cells that further differentiate into the specialized cell types (Jakob et al. 2004). In contrary to animals fed by *P. cruentum,* exposure to 9-*cis*-RA was not associated with the development of the balloon-like phenotype and animals survived more than 250 h (**Supplementary Fig. S3**). In contrast to control animals which started to proliferate after 100 h, animals exposed to 9-*cis*-RA did not proliferate between 90 and 280 h of subsequent culture (**Fig. 8 D**) suggesting that 9-*cis*-RA interferes with animal response to specific food and processes necessary for animal growth and propagation. The growth arrest of *T. adhaerens* caused by 9-*cis*-RA was reverted by addition of *Porphyridium cruentum* indicating that a specific food constituent rather than food availability interferes with 9-*cis*-RA regulatory potential (**Supplementary Fig S4**).

DISCUSSION

T. adhaerens is probably the closest living species to basal metazoans with only four NRs

Trichoplax adhaerens is an especially interesting species from an evolutionary perspective. It shows the most primitive metazoan planar body arrangement known with a simple

480 dorsal-ventral polarity, the establishment of which is one of the most ancient events in evolution
481 of animal symmetries (Smith et al. 1995; Stein & Stevens 2014). The Placozoa are disposed with
482 only a few (probably six) morphologically recognizable cell types (Jakob et al. 2004; Smith et
483 al. 2014).

484 In strong contrast to this, the *T. adhaerens* genome shows larger blocks of conserved
485 synteny relative to the human genome than flies or nematodes (Srivastava et al. 2008). Genome
486 analyses indicate that Placozoa are basal relative to Bilateria as well as all other diploblastic
487 phyla (Schierwater et al. 2009), but all kinds of different views are also discussed (reviewed in
488 (Schierwater et al. 2016)).

489 In concordance with this, its genome contains four (Srivastava et al. 2008) rather than 17
490 NRs, which can be found in the cnidarian *Nematostella vectensis* (Reitzel & Tarrant 2009). Even
491 though it has been proposed that Placozoa lost representatives of NR6 (SF1/GCNF), TR2/TR4 of
492 the NR2 subfamily and invertebrate specific nuclear receptors (INR, clade of invertebrate-only
493 nuclear receptors with no standard nomenclature) NR1/NR4 (Bridgham et al. 2010). The reasoning
494 in this direction depends on the assumed phylogenic position of the phylum Placozoa.

495 The four NRs found in the genome of *T. adhaerens* are relatively highly related to their
496 vertebrate counterparts, RXR (NR2B), HNF4 (NR2A), COUP-TF (NR2F) and ERR (NR3B)
497 (Srivastava et al. 2008). Among them, *T. adhaerens* RXR and HNF4 show the highest degree of
498 identity in protein sequence and the relatedness of *T. adhaerens* RXR (TaRXR) to human RXR is
499 similar to that of *Tripedalia cystophora* RXR (jRXR) (Kostrouch et al. 1998), which has also been
500 shown to bind 9-*cis*-RA at nanomolar concentrations. These results suggest that TaRXR is
501 structurally and also functionally very closely related to its vertebrate counterparts, most probably
502 representing the most basal liganded NR of all Metazoa known today.

503

504

505 ## *T. adhaerens* RXR binds preferentially 9-*cis*-RA

506 By using a radioactively labelled ligand, we could demonstrate that the RXR orthologue
507 in *T. adhaerens* binds 9-*cis*-RA with very high affinity and shows a strong binding preference to
508 9-*cis*-RA over all-*trans*-RA similarly, as is the case in vertebrate RXRs (Allenby et al. 1993) and
509 the cnidarian TcRXR (Kostrouch et al. 1998).

510

511

512 ## 9-*cis*-RA affects the expression of a *T. adhaerens* orthologue of a conserved
513 ## metabolically active enzyme, L-malate-NADP$^+$ oxidoreductase

514

515 A biological role of 9-*cis*-RA binding with high affinity to the placozoan RXR receptor is
516 supported by our *in vivo* experiments. In the search for genes that may be under the regulation of
517 TaRXR, we identified a putative orthologue of vertebrate L-malate-NADP$^+$ oxidoreductase (EC
518 1.1.1.40) and analyzed the effect of 9-*cis*-RA or all-*trans*-RA on its expression. In agreement with
519 our binding experiments, we observed induction of this enzyme's expression at low nanomolar
520 concentrations of 9-cis-RA (1 to 10 nM). Interestingly, higher concentrations of 9-*cis*-RA (30 nM)
521 had a smaller effect on expression and all-*trans*-RA had no effect up to 30 nM concentrations. A
522 plausible explanation for this could be that 9-*cis*-RA also acts as a ligand for other *T. adhaerens*
523 NRs which may have an opposite effect on the expression of L-malate-NADP$^+$ oxidoreductase.
524 Furthermore, 9-*cis*-RA could act as a supranatural ligand and the continuous occupation of TaRXR

525 by this high affinity ligand may interfere with the normal function of the receptor within the
526 transcription initiation machinery.

527 In mammals, regulation of malic enzyme expression is mediated by a thyroid hormone
528 receptor (TR) – RXR heterodimer (Dozin et al. 1985a; Dozin et al. 1985b; Petty et al. 1989; Petty
529 et al. 1990). By showing a 9-*cis*-RA dependent change in the expression of the likely placozoan
530 malic enzyme orthologue *in vivo*, we provide indirect evidence of a conserved RXR mediated
531 regulation of gene expression. Although the expression of L-malate-$NADP^+$ oxidoreductase in
532 mammals is usually used as a factor reflecting regulation by thyroid hormone (Dozin et al. 1986),
533 it has also been shown that its cell-type associated differences depend on the expression level of
534 RXR alpha (Hillgartner et al. 1992; Fang & Hillgartner 2000) suggesting that regulation by RXR
535 has been conserved throughout metazoan evolution while additional regulation via thyroid
536 hormone represents an innovation of Bilateria (Wu et al. 2007).

537

538 **NRs form a network responding to 9-*cis*-RA**

539 Since autoregulation and cross-regulation of NRs by their specific ligands is well
540 documented for a large number of nuclear receptors (Tata 1994), we searched if 9-*cis*-RA affects
541 the expression of TaRXR mRNA relative to the other *T. adhaerens* NRs. Our *in vivo* experiments
542 showed not only effects on specific gene expression in response to very low concentrations of 9-
543 *cis*-RA (at 1 or 3 nM), but also an additional dose-dependent reverse effect of higher
544 concentrations. This is likely to be in line with our binding experiments that suggested the
545 possibility of an additional binding site or sites with higher capacity and lower affinity. We also
546 cannot rule out that higher concentrations of 9-*cis*-RA affect some of the three remaining *T.*

547 *adhaerens* NRs. Nevertheless, an inhibitory effect of 9-*cis*-RA on the expression of its cognate
548 receptor at the protein level (through protein degradation) was reported (Nomura et al. 1999).
549 Although it is not clear if 9-*cis*-RA is the natural ligand for RXRs (Wolf 2006; Ruhl et al.
550 2015) conserved in all metazoan phyla studied to date, we show not only that 9-*cis*-RA binds
551 TaRXR with nanomolar affinity but also positively regulates its expression, which resembles auto-
552 activation of several NRs in vertebrates [e.g. ER and TR (Tata 1994; Bagamasbad & Denver
553 2011)]. Furthermore, three out of four NRs constituting the NR complement in *T. adhaerens*
554 respond to treatment by 9-*cis*-RA at transcriptional level. Two NRs, RXR itself and ERR respond
555 positively to nanomolar concentrations of 9-*cis*-RA, while COUP-TF, which often acts as an
556 inhibitor of specific gene expression (Tran et al. 1992), is regulated negatively by 9-*cis*-RA.
557 COUP-TF was recently shown to be inactivated by small hydrophobic molecules (Le Guevel et al.
558 2017). The regulatory connections of *T. adhaerens* NRs places the autoregulation and cross-
559 regulation of NRs to the base of metazoan evolution. The proposed regulatory network of *T.*
560 *adhaerens* NRs is schematically represented in **Supplementary Fig. S5**.
561

562 ## Food composition rather than quantity affects phenotype of *T. adhaerens*

563 At first glance, *T. adhaerens* seems to benefit from any source of biological material on
564 surfaces that can be digested and absorbed by its digestive system (e.g. aquarium microorganisms
565 and detritus). Feeding with certain live microorganisms in laboratory cultures, however,
566 dramatically changes the dynamics of *T. adhaerens* cultures, such as shape, size, color, body
567 transparency, growth and divisions of the animal. For example, we observed poor growth and
568 reproduction rates of *T. adhaerens* fed solely on *Chlorella sp.* even at a relatively high density. In

569 contrast, cultures fed with red pigment containing *Rhodomonas salina* showed much faster
570 proliferation and led, in part, to the formation of giant animals, seeming to halt their division.
571 Despite *Porphyridium cruentum* containing similar pigments as *Rhodomonas*, such as
572 phycoerythrin, cultures grown with *Porphyridium* as the main nutrient source did not show
573 phenotypical abnormalities but the addition of it to a culture with *Chlorella* and *Rhodomonas*
574 resulted in an additive effect on reproduction rate.

575 Even though the growth of *T. adhaerens* seems to follow a simple program, it is likely to
576 require strict regulatory mechanisms. Formation of specific cellular types is connected with
577 phenotypic appearance of animals possessing larger proportions of certain cells, e.g. upper
578 epithelium in balloon-like animals or larger proportion of peripheral regions containing stem cell-
579 like cells in narrow or prolonged animals. Analysis of circularity as a measure of location specific
580 cellular proliferation is in concordance with the observed culture characteristics and shows that
581 lower isoperimetric values (less 'roundness') indicate higher reproduction rates.

582 Our experiments provide evidence that food composition is more important for *T.*
583 *adhaerens* growth and propagation than its quantity, which is in line with the recent finding of
584 phosphate and nitrate playing important roles determining the distribution of placozoans around
585 the globe (Paknia & Schierwater 2015). It indicates that food constituents, especially those present
586 in the algae containing phycobilin based red pigments like *Rhodomonas salina* and *Porphyridium*
587 *cruentum* might possess hormone-like molecules or molecules resulting in hormone-like
588 metabolites in *T. adhaerens* that act through the NR complement and, indeed, analysis of NRs in
589 differently sized animals indicates impact of food composition on NR expression.

590 The high sensitivity of *T. adhaerens* to 9-*cis*-RA reflected by the transcriptional response
591 to low nanomolar concentrations of 9-*cis*-RA but not all-*trans*-RA and the interference of 3.3 nM

9-*cis*-RA with the animal response to feeding together with the high affinity binding of 9-*cis*-RA by TaRXR suggests that the response of *T. adhaerens* to 9-*cis*-RA is mediated by TaRXR. It cannot be excluded that other *T. adhaerens* NRs, especially TaCOUP-TF and possibly also TaHNF4 may also be affected by 9-*cis*-RA. It seems possible that 9-*cis*-RA or similarly shaped molecules may be present in *T. adhaerens* food or can be formed from retinoids and other molecular components of food. Our data indicate that the sensitivity of *T. adhaerens* to 9-*cis*-RA depends on the actual feeding conditions and animal growth. There are several possible scenarios that may explain the high sensitivity of *T. adhaerens* to 9-*cis*-RA. The activation of RXR by 9-*cis*-RA or similar compounds has been documented in vertebrates (Allenby et al. 1993; Ruhl et al. 2015; de Lera et al. 2016). The observation of 9-*cis*-RA induced growth arrest is similar as data reported on mammalian cells (e.g. (Wente et al. 2007)) however the concentration of 9-*cis*-RA used in our experiments is approximately 30 to 3000 times lower than the levels reported in most mammalian systems. It seems likely that very low concentrations of natural ligands including 9-*cis*-RA or similarly shaped molecules or other molecules present in the algal food or produced from algal food as metabolites in *T. adhaerens* regulate the gene expression via RXR in *T. adhaerens*. This may be connected with *T. adhaerens* strong response to light exposure visible as coordinated relocations of animals inside laboratory culture containers and a strong influence of annual seasons on *T. adhaerens* propagation rates observed in laboratories localized in temperate geographical zones. The 9-*cis* conformation of RA is not only sensitive to light exposure with its reversal to all-trans conformation but it can also be formed by specific UV irradiation from all-trans conformation up to 10% as shown by Dr. Hans Cahnmann (Cahnmann 1995).

Chlorophyll hydrophobic side chain which anchors the molecule to the chloroplast thylakoid membrane is metabolized to phytol that was shown to act as an RXR agonist (Kitareewan

et al. 1996). Other molecules called rexinoids, which often contain aromatic rings in their structure act as RXR agonists or antagonists (Dawson & Xia 2012). Lately, another group of ligands called organotins was shown to affect regulation by RXR (le Maire et al. 2009). It has been proposed that RXRs can bind a larger group of polyunsaturated fatty acids (docosahexaenoic acid and arachidonic acid) and act as their sensors (de Urquiza et al. 2000; Lengqvist et al. 2004).

When viewed together, our work shows the presence of 9-*cis*-RA binding RXR in Placozoa and argues for the existence of ligand regulated NRs at the base of metazoan evolution. Our observations suggest the existence an endocrine-like regulatory network of NRs in *T. adhaerens* (schematically represented in **Supplementary Fig. S5**). Endocrine, hormone – receptor regulations involving NRs may be viewed as specialized, very powerful yet not prevailing regulations transmitted by NRs. Increasingly bigger numbers of non-hormonal ligands originating in environment, food or metabolism are emerging as regulatory molecules of NRs (Holzer et al. 2017). Our data suggest that non-hormonal, environment and food derived ligands are likely to be the first or very early ligands regulating the metazoan response to food availability and orchestrating growth of basal metazoans and necessary differentiation to specialized cell types. In this sense, NRs in *T. adhaerens* represent an endocrine-like system of ancestor NRs.

This work suggests that ligand regulated RXR is involved in the coordination of animal growth and development throughout the metazoan evolution. This also suggests that the regulation by liganded NRs evolved as an evolutionary need connected with heterotrophy and multicellularity.

In fact, despite fragments of NR domains being found in prokaryotes, no single full sized NR has been discovered in bacteria or archaea and the closest known relatives to metazoans, unicellular and colonial Choanoflagellates, lack nuclear receptors, as well as genes of several other

638 regulatory pathways (King et al. 2008). On the other hand in fungi, the sister group of Holozoa,
639 (Shalchian-Tabrizi et al. 2008) transcription factors surprisingly similar to metazoan NRs evolved
640 independently possibly for the regulation of metabolism and response to xenobiotics (Thakur et
641 al. 2008; Naar & Thakur 2009). Thus, the evolution of NRs seems to be associated with two key
642 evolutionary features of metazoans: multicellularity and heterotrophy.

643 Ctenophores, a possible sister phylum to *Cnidaria*, do not contain classical NRs featuring
644 both mechanistically critical domains of NRs, the DNA binding and ligand binding domains.
645 Nevertheless, the ctenophore *Mnemiopsis* contains two orthologues of NR2A (HNF4) that lack the
646 DNA binding domain (Reitzel et al. 2011).

647 Our observations of the exceptionally high sensitivity of *T. adhaerens* to 9-*cis*-RA imply
648 the possibility that the originally very strong regulations mediated by NRs might have been
649 softened or inhibited by additionally evolved mechanisms. To our knowledge, there are no reports
650 of 100% lethal effects of exposure to low nanomolar levels of 9-*cis*-RA in any metazoan organism.
651 It can be speculated that these mechanisms were likely to evolve to modulate 9-*cis*-RA's or similar
652 ligand's regulatory potential further and might involve stronger regulations by heterodimerization
653 partners of RXR and enzymatic or transport mechanisms regulating the availability of ligands in
654 cells and tissues of more recent Metazoa.

655 In conclusion, the presence of functional nuclear receptors in *T. adhaerens* and their
656 proposed regulatory network support the hypothesis of a basic regulatory mechanism by NRs,
657 which may have been subspecialized with the appearance of new NRs in order to cope with new
658 environmental and behavioral challenges during the course of early metazoan evolution and
659 developmental regulatory needs of increasingly more complex metazoan species.

660

Supplementary information

Unprocessed data and additional information: Supplementary File S4 – qPCR and ddPCR data, Supplementary File S5 – part A – Images of *T. adhaerens* incubated with specific algal food, Supplementary File S5 – part B – Images of *T. adhaerens* incubated with specific algal food, Supplementary File S6 Image analysis of T. adhaerens incubated with specific algal food, Supplementary File S10 - Images of control and 9-cis-RA treated Trichoplax fed by Porphyridium cruentum at time 0 and after 24 h, Supplmentary File S12 - Additional information concerning the analysis of the binding of 9-*cis*-RA to *Trichoplax adhaerens* RXR. Assessment of the quality of GST-RXR by polyacrylamide gel electrophoresis.

Acknowledgements

The databases of NCBI (Coordinators 2017) and Joint Genome Institute of United States Department of Energy (http://jgi.doe.gov/) (Nordberg et al. 2014) provided bioinformatics support for this study.

References

Allenby G, Bocquel MT, Saunders M, Kazmer S, Speck J, Rosenberger M, Lovey A, Kastner P, Grippo JF, Chambon P, and Levin AA. 1993. Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. *Proc Natl Acad Sci U S A* 90:30-34.

Bagamasbad P, and Denver RJ. 2011. Mechanisms and significance of nuclear receptor auto- and cross-regulation. *Gen Comp Endocrinol* 170:3-17. 10.1016/j.ygcen.2010.03.013

Baker ME. 2008. Trichoplax, the simplest known animal, contains an estrogen-related receptor but no estrogen receptor: Implications for estrogen receptor evolution. *Biochem Biophys Res Commun* 375:623-627.

Bell EA, Boehnke P, Harrison TM, and Mao WL. 2015. Potentially biogenic carbon preserved in a 4.1 billion-year-old zircon. *Proc Natl Acad Sci U S A* 112:14518-14521. 10.1073/pnas.1517557112

Bridgham JT, Eick GN, Larroux C, Deshpande K, Harms MJ, Gauthier ME, Ortlund EA, Degnan BM, and Thornton JW. 2010. Protein evolution by molecular tinkering: diversification

691 of the nuclear receptor superfamily from a ligand-dependent ancestor. *PLoS Biol* 8.
692 10.1371/journal.pbio.1000497
693 Cahnmann HJ. 1995. A fast photoisomerization method for the preparation of tritium-labeled
694 9-cis-retinoic acid of high specific activity. *Anal Biochem* 227:49-53.
695 10.1006/abio.1995.1251
696 Coordinators NR. 2017. Database Resources of the National Center for Biotechnology
697 Information. *Nucleic Acids Res* 45:D12-D17. 10.1093/nar/gkw1071
698 Dawson MI, and Xia ZB. 2012. The retinoid X receptors and their ligands. *Biochimica Et*
699 *Biophysica Acta-Molecular and Cell Biology of Lipids* 1821:21-56.
700 10.1016/j.bbalip.2011.09.014
701 de Lera AR, Krezel W, and Ruhl R. 2016. An Endogenous Mammalian Retinoid X Receptor
702 Ligand, At Last! *ChemMedChem* 11:1027-1037. 10.1002/cmdc.201600105
703 de Urquiza AM, Liu S, Sjoberg M, Zetterstrom RH, Griffiths W, Sjovall J, and Perlmann T. 2000.
704 Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science*
705 290:2140-2144.
706 Dozin B, Cahnmann HJ, and Nikodem VM. 1985a. Identification of thyroid hormone receptors
707 in rat liver nuclei by photoaffinity labeling with L-thyroxine and triiodo-L-thyronine.
708 *Biochemistry* 24:5197-5202.
709 Dozin B, Magnuson MA, and Nikodem VM. 1985b. Tissue-specific regulation of two functional
710 malic enzyme mRNAs by triiodothyronine. *Biochemistry* 24:5581-5586.
711 Dozin B, Magnuson MA, and Nikodem VM. 1986. Thyroid hormone regulation of malic
712 enzyme synthesis. Dual tissue-specific control. *J Biol Chem* 261:10290-10292.
713 Egea PF, Klaholz BP, and Moras D. 2000. Ligand-protein interactions in nuclear receptors of
714 hormones. *FEBS letters* 476:62-67.
715 Escriva H, Bertrand S, and Laudet V. 2004. The evolution of the nuclear receptor superfamily.
716 *Essays Biochem* 40:11-26.
717 Escriva H, Delaunay F, and Laudet V. 2000. Ligand binding and nuclear receptor evolution.
718 *Bioessays* 22:717-727. 10.1002/1521-1878(200008)22:8<717::AID-BIES5>3.0.CO;2-
719 I
720 Escriva H, Langlois MC, Mendonca RL, Pierce R, and Laudet V. 1998. Evolution and
721 diversification of the nuclear receptor superfamily. *Ann N Y Acad Sci* 839:143-146.
722 Escriva H, Safi R, Hanni C, Langlois MC, Saumitou-Laprade P, Stehelin D, Capron A, Pierce R,
723 and Laudet V. 1997. Ligand binding was acquired during evolution of nuclear
724 receptors. *Proc Natl Acad Sci U S A* 94:6803-6808.
725 Fang X, and Hillgartner FB. 2000. Alterations in retinoid X receptor-alpha expression
726 contribute to cell-type dependent differences in thyroid hormone regulation of malic
727 enzyme transcription. *Mol Cell Endocrinol* 164:41-52.
728 Forman BM, and Samuels HH. 1990a. Dimerization among nuclear hormone receptors. *New*
729 *Biol* 2:587-594.
730 Forman BM, and Samuels HH. 1990b. Interactions among a subfamily of nuclear hormone
731 receptors: the regulatory zipper model. *Mol Endocrinol* 4:1293-1301. 10.1210/mend-
732 4-9-1293
733 Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, and Gascuel O. 2010. New
734 algorithms and methods to estimate maximum-likelihood phylogenies: assessing the
735 performance of PhyML 3.0. *Syst Biol* 59:307-321. 10.1093/sysbio/syq010

736 Gutierrez-Mazariegos J, Schubert M, and Laudet V. 2014. Evolution of retinoic acid receptors
737 and retinoic acid signaling. *Subcell Biochem* 70:55-73. 10.1007/978-94-017-9050-
738 5_4
739 Hillgartner FB, Chen W, and Goodridge AG. 1992. Overexpression of the alpha-thyroid
740 hormone receptor in avian cell lines. Effects on expression of the malic enzyme gene
741 are selective and cell-specific. *J Biol Chem* 267:12299-12306.
742 Holzer G, Markov GV, and Laudet V. 2017. Evolution of Nuclear Receptors and Ligand
743 Signaling: Toward a Soft Key-Lock Model? *Curr Top Dev Biol* 125:1-38.
744 10.1016/bs.ctdb.2017.02.003
745 Issemann I, and Green S. 1990. Activation of a member of the steroid hormone receptor
746 superfamily by peroxisome proliferators. *Nature* 347:645-650. 10.1038/347645a0
747 Jakob W, Sagasser S, Dellaporta S, Holland P, Kuhn K, and Schierwater B. 2004. The Trox-2
748 Hox/ParaHox gene of Trichoplax (Placozoa) marks an epithelial boundary. *Dev Genes*
749 *Evol* 214:170-175.
750 Kana R, Kotabova E, Lukes M, Papacek S, Matonoha C, Liu LN, Prasil O, and Mullineaux CW.
751 2014. Phycobilisome Mobility and Its Role in the Regulation of Light Harvesting in
752 Red Algae. *Plant Physiol* 165:1618-1631. 10.1104/pp.114.236075
753 Kana R, Kotabova E, Sobotka R, and Prasil O. 2012. Non-photochemical quenching in
754 cryptophyte alga Rhodomonas salina is located in chlorophyll a/c antennae. *PLoS One*
755 7:e29700.
756 Kiefer JC. 2006. Emerging developmental model systems. *Dev Dyn* 235:2895-2899.
757 King N, Westbrook MJ, Young SL, Kuo A, Abedin M, Chapman J, Fairclough S, Hellsten U, Isogai
758 Y, Letunic I, Marr M, Pincus D, Putnam N, Rokas A, Wright KJ, Zuzow R, Dirks W, Good
759 M, Goodstein D, Lemons D, Li W, Lyons JB, Morris A, Nichols S, Richter DJ, Salamov A,
760 Sequencing JG, Bork P, Lim WA, Manning G, Miller WT, McGinnis W, Shapiro H, Tjian
761 R, Grigoriev IV, and Rokhsar D. 2008. The genome of the choanoflagellate Monosiga
762 brevicollis and the origin of metazoans. *Nature* 451:783-788. 10.1038/nature06617
763 Kitareewan S, Burka LT, Tomer KB, Parker CE, Deterding LJ, Stevens RD, Forman BM, Mais
764 DE, Heyman RA, McMorris T, and Weinberger C. 1996. Phytol metabolites are
765 circulating dietary factors that activate the nuclear receptor RXR. *Mol Biol Cell*
766 7:1153-1166.
767 Kostrouch Z, Kostrouchova M, Love W, Jannini E, Piatigorsky J, and Rall JE. 1998. Retinoic
768 acid X receptor in the diploblast, Tripedalia cystophora. *Proc Natl Acad Sci U S A*
769 95:13442-13447.
770 Kostrouch Z, Kostrouchova M, and Rall JE. 1995. Steroid/thyroid hormone receptor genes in
771 Caenorhabditis elegans. *Proc Natl Acad Sci U S A* 92:156-159.
772 Kostrouchova M, and Kostrouch Z. 2015. Nuclear receptors in nematode development:
773 Natural experiments made by a phylum. *Biochim Biophys Acta* 1849:224-237.
774 10.1016/j.bbagrm.2014.06.016
775 Kumar R, and Thompson EB. 1999. The structure of the nuclear hormone receptors. *Steroids*
776 64:310-319.
777 Laudet V. 1997. Evolution of the nuclear receptor superfamily: early diversification from an
778 ancestral orphan receptor. *J Mol Endocrinol* 19:207-226.
779 Le Guevel R, Oger F, Martinez-Jimenez CP, Bizot M, Gheeraert C, Firmin F, Ploton M, Kretova
780 M, Palierne G, Staels B, Barath P, Talianidis I, Lefebvre P, Eeckhoute J, and Salbert G.

2017. Inactivation of the Nuclear Orphan Receptor COUP-TFII by Small Chemicals. *ACS Chem Biol*. 10.1021/acschembio.6b00593

le Maire A, Grimaldi M, Roecklin D, Dagnino S, Vivat-Hannah V, Balaguer P, and Bourguet W. 2009. Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors. *EMBO Rep* 10:367-373. 10.1038/embor.2009.8

Lengqvist J, Mata De Urquiza A, Bergman AC, Willson TM, Sjovall J, Perlmann T, and Griffiths WJ. 2004. Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor alpha ligand-binding domain. *Mol Cell Proteomics* 3:692-703. 10.1074/mcp.M400003-MCP200

Letunic I, Doerks T, and Bork P. 2015. SMART: recent updates, new developments and status in 2015. *Nucleic Acids Res* 43:D257-260. 10.1093/nar/gku949

Markov GV, and Laudet V. 2011. Origin and evolution of the ligand-binding ability of nuclear receptors. *Mol Cell Endocrinol* 334:21-30. 10.1016/j.mce.2010.10.017

Naar AM, and Thakur JK. 2009. Nuclear receptor-like transcription factors in fungi. *Genes Dev* 23:419-432. 10.1101/gad.1743009

Nomura Y, Nagaya T, Hayashi Y, Kambe F, and Seo H. 1999. 9-cis-retinoic acid decreases the level of its cognate receptor, retinoid X receptor, through acceleration of the turnover. *Biochem Biophys Res Commun* 260:729-733. 10.1006/bbrc.1999.0969

Nordberg H, Cantor M, Dusheyko S, Hua S, Poliakov A, Shabalov I, Smirnova T, Grigoriev IV, and Dubchak I. 2014. The genome portal of the Department of Energy Joint Genome Institute: 2014 updates. *Nucleic Acids Res* 42:D26-31. 10.1093/nar/gkt1069

Nowickyj SM, Chithalen JV, Cameron D, Tyshenko MG, Petkovich M, Wyatt GR, Jones G, and Walker VK. 2008. Locust retinoid X receptors: 9-Cis-retinoic acid in embryos from a primitive insect. *Proc Natl Acad Sci U S A* 105:9540-9545. 10.1073/pnas.0712132105

Paknia O, and Schierwater B. 2015. Global Habitat Suitability and Ecological Niche Separation in the Phylum Placozoa. *PLoS One* 10:e0140162. 10.1371/journal.pone.0140162

Petty KJ, Desvergne B, Mitsuhashi T, and Nikodem VM. 1990. Identification of a thyroid hormone response element in the malic enzyme gene. *J Biol Chem* 265:7395-7400.

Petty KJ, Morioka H, Mitsuhashi T, and Nikodem VM. 1989. Thyroid hormone regulation of transcription factors involved in malic enzyme gene expression. *J Biol Chem* 264:11483-11490.

Reitzel AM, Pang K, Ryan JF, Mullikin JC, Martindale MQ, Baxevanis AD, and Tarrant AM. 2011. Nuclear receptors from the ctenophore Mnemiopsis leidyi lack a zinc-finger DNA-binding domain: lineage-specific loss or ancestral condition in the emergence of the nuclear receptor superfamily? *Evodevo* 2:3. 10.1186/2041-9139-2-3

Reitzel AM, and Tarrant AM. 2009. Nuclear receptor complement of the cnidarian Nematostella vectensis: phylogenetic relationships and developmental expression patterns. *BMC Evol Biol* 9:230. 10.1186/1471-2148-9-230

Robinson-Rechavi M, Escriva Garcia H, and Laudet V. 2003. The nuclear receptor superfamily. *J Cell Sci* 116:585-586.

Ruhl R, Krzyzosiak A, Niewiadomska-Cimicka A, Rochel N, Szeles L, Vaz B, Wietrzych-Schindler M, Alvarez S, Szklenar M, Nagy L, de Lera AR, and Krezel W. 2015. 9-cis-13,14-Dihydroretinoic Acid Is an Endogenous Retinoid Acting as RXR Ligand in Mice. *PLoS Genet* 11:e1005213. 10.1371/journal.pgen.1005213

826 Schierwater, Holland PWH, Miller DJ, Stadler PF, Wiegmann BM, Worheide G, Wray GA, and
827 R D. 2016. Never Ending Analysis of a Century Old Evolutionary Debate: "Unringing"
828 the Urmetazoon Bell. *Front Ecol Evol,* 4:5. 10.3389/fevo.2016.00005
829 Schierwater B, Kolokotronis SO, Eitel M, and DeSalle R. 2009. The Diploblast-Bilateria Sister
830 hypothesis: parallel revolution of a nervous systems may have been a simple step.
831 *Commun Integr Biol* 2:403-405.
832 Schultz J, Milpetz F, Bork P, and Ponting CP. 1998. SMART, a simple modular architecture
833 research tool: identification of signaling domains. *Proc Natl Acad Sci U S A* 95:5857-
834 5864.
835 Shalchian-Tabrizi K, Minge MA, Espelund M, Orr R, Ruden T, Jakobsen KS, and Cavalier-Smith
836 T. 2008. Multigene phylogeny of choanozoa and the origin of animals. *PLoS One*
837 3:e2098. 10.1371/journal.pone.0002098
838 Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M,
839 Soding J, Thompson JD, and Higgins DG. 2011. Fast, scalable generation of high-quality
840 protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 7:539.
841 10.1038/msb.2011.75
842 Smith CL, Giordano H, Schwartz M, and DeLotto R. 1995. Spatial regulation of Drosophila
843 snake protease activity in the generation of dorsal-ventral polarity. *Development*
844 121:4127-4135.
845 Smith CL, Varoqueaux F, Kittelmann M, Azzam RN, Cooper B, Winters CA, Eitel M, Fasshauer
846 D, and Reese TS. 2014. Novel cell types, neurosecretory cells, and body plan of the
847 early-diverging metazoan Trichoplax adhaerens. *Curr Biol* 24:1565-1572.
848 10.1016/j.cub.2014.05.046
849 Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, Kawashima T, Kuo A, Mitros T,
850 Salamov A, Carpenter ML, Signorovitch AY, Moreno MA, Kamm K, Grimwood J,
851 Schmutz J, Shapiro H, Grigoriev IV, Buss LW, Schierwater B, Dellaporta SL, and
852 Rokhsar DS. 2008. The Trichoplax genome and the nature of placozoans. *Nature*
853 454:955-960.
854 Stein DS, and Stevens LM. 2014. Maternal control of the Drosophila dorsal-ventral body axis.
855 *Wiley Interdiscip Rev Dev Biol* 3:301-330. 10.1002/wdev.138
856 Tata JR. 1994. Autoregulation and crossregulation of nuclear receptor genes. *Trends*
857 *Endocrinol Metab* 5:283-290.
858 Thakur JK, Arthanari H, Yang F, Pan SJ, Fan X, Breger J, Frueh DP, Gulshan K, Li DK, Mylonakis
859 E, Struhl K, Moye-Rowley WS, Cormack BP, Wagner G, and Naar AM. 2008. A nuclear
860 receptor-like pathway regulating multidrug resistance in fungi. *Nature* 452:604-609.
861 10.1038/nature06836
862 Tran P, Zhang XK, Salbert G, Hermann T, Lehmann JM, and Pfahl M. 1992. COUP orphan
863 receptors are negative regulators of retinoic acid response pathways. *Mol Cell Biol*
864 12:4666-4676.
865 Wente W, Brenner MB, Zitzer H, Gromada J, and Efanov AM. 2007. Activation of liver X
866 receptors and retinoid X receptors induces growth arrest and apoptosis in insulin-
867 secreting cells. *Endocrinology* 148:1843-1849. 10.1210/en.2006-1247
868 Wolf G. 2006. Is 9-cis-retinoic acid the endogenous ligand for the retinoic acid-X receptor?
869 *Nutr Rev* 64:532-538.

870 Wu W, Niles EG, and LoVerde PT. 2007. Thyroid hormone receptor orthologues from
871 invertebrate species with emphasis on Schistosoma mansoni. *BMC Evol Biol* 7:150.
872 10.1186/1471-2148-7-150
873

Figure 1

Multiple sequence alignment of selected metazoan homologues of RXR compared with TaRXR.

Aligned with ClustalO, amino acid residue types colored according to Clustal scheme in Jalview, * indicates DBD footprint residues, # LBD footprint residues. Black box shows the DBD, red box represents the LBD. Sequences from top to bottom (organism, identifier): *Trichoplax adhaerens*, TaRXR ID 53515; *Homo sapiens*, sp|P19793|RXRA_HUMAN; *Homo sapiens*, sp|P28702|RXRB_HUMAN; *Homo sapiens*, sp|P48443|RXRG_HUMAN; *Xenopus laevis*, RXR alpha, sp|P51128|RXRA_XENLA; *Aurelia aurita*, RXR, tr|S5ZWR0|S5ZWR0_AURAU Retinoid X receptor; *Tripedalia cystophora*, RXR, tr|O96562|O96562_TRICY Retinoic acid X receptor; *Drosophila melanogaster*, USP, sp|P20153|USP_DROME. DNA binding domain (DBD, red line), Ligand binding domain (LBD, green line), dimerization domain (yellow line) and amino acid residues critical for 9-*cis*-RA binding (conserved – red rectangles, not conserved – pink rectangles) are indicated. Readers with specific color preferences may download the compared sequences (**Supplementary File S1**) and create the Clustal scheme with different color specifications using the Jalview program (http://www.jalview.org/).

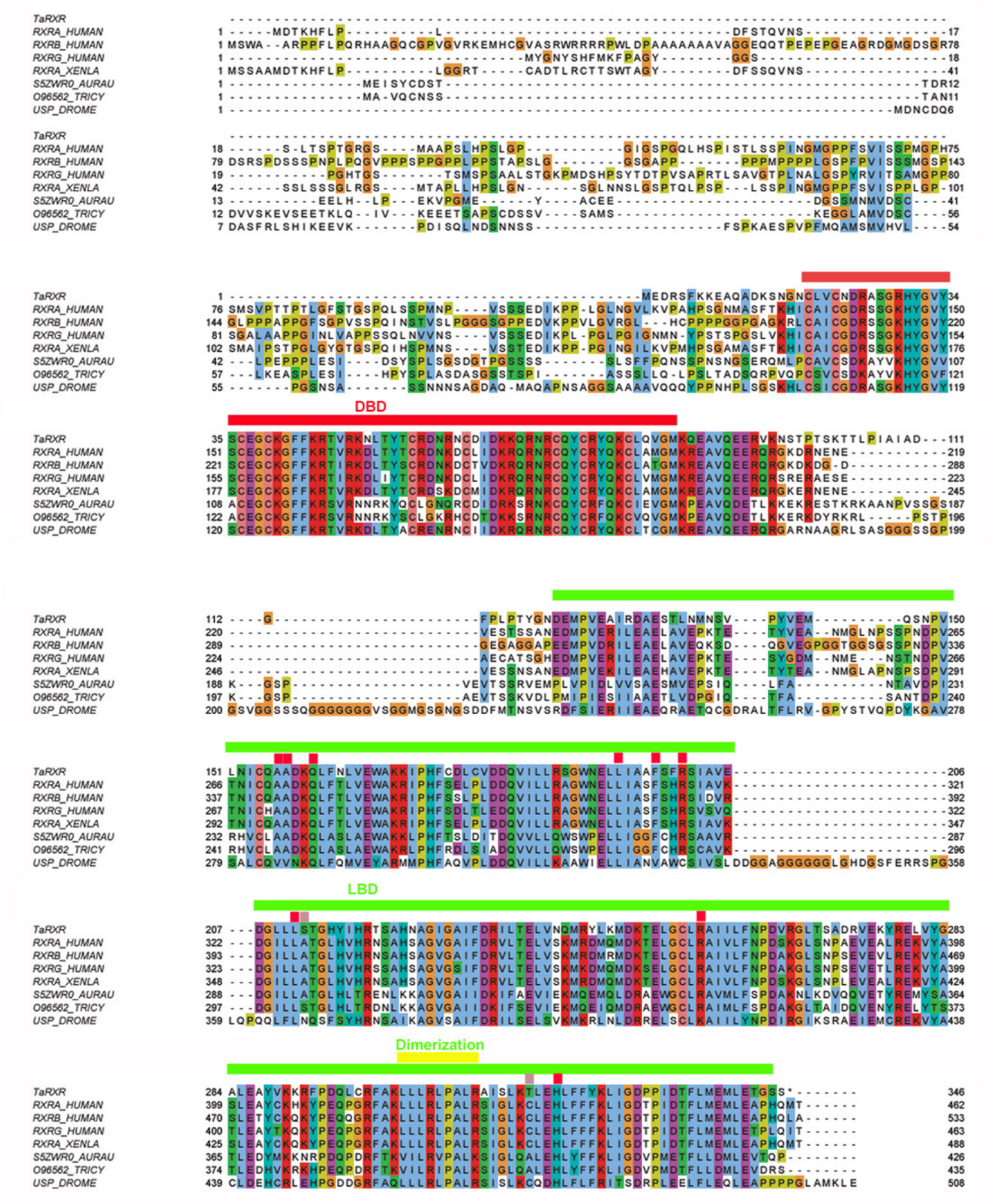

DBD
LBD
Dimerization

Figure 2

Binding of retinoic acids to TaRXR.

A – Single point analysis of binding preference of *T. adhaerens* RXR (thrombin cleaved) to ^{3}H-labelled 9-*cis*-RA over all-*trans*-RA. Radioactive 9-*cis*-RA (9-*cis*-RA*) binds at a concentration of 4 nM to 200 nanograms of *T. adhaerens* RXR. 200-fold excess of unlabeled *9-cis*-RA displaces more than 80 % of labeled *9-cis*-RA from binding to *T. adhaerens* RXR (9-*cis*-RA* + 9-*cis*-RA) while the same molar excess of all-*trans*-RA (9-*cis*-RA* + AT-RA) which is likely to contain approximately 1% spontaneously isomerized 9-*cis*- RA, competes away less than 50 % of bound ^{3}H-labeled 9-*cis*-RA. Radioactive ^{3}H-labeld all-*trans*-RA (AT-RA*) at identical conditions binds only slightly more than the observed non-specific binding. This interaction is not displaced by the excess of non-labeled 9-*cis*-RA (AT-RA* + 9-*cis*-RA) nor non-labeled all-*trans*-RA (AT-RA* + AT-RA). Results are expressed as a ratio of the radioactivity bound to TaRXR / total radioactivity used for the binding at the given condition. B – Analysis of binding properties of *T. adhaerens* RXR (in the form of GST-TaRXR) to ^{3}H-labelled 9-*cis*-RA and ^{3}H-labelled all-*trans*-RA. The experiment differs from the experiment shown in A in 5-fold greater amount of radioactive all-*trans*-RA (and therefore only 40-fold excess of non-radioactive competitors). The experiment shows identical binding properties of GST-TaRXR as those observed with thrombin cleaved TaRXR. C – Kinetic analysis of binding of ^{3}H-labeled 9-*cis*-RA to *T. adhaerens* RXR prepared as GST-fusion protein (GST-TaRXR). The plateau is reached at around 3 to 5 x10^{-9} M.

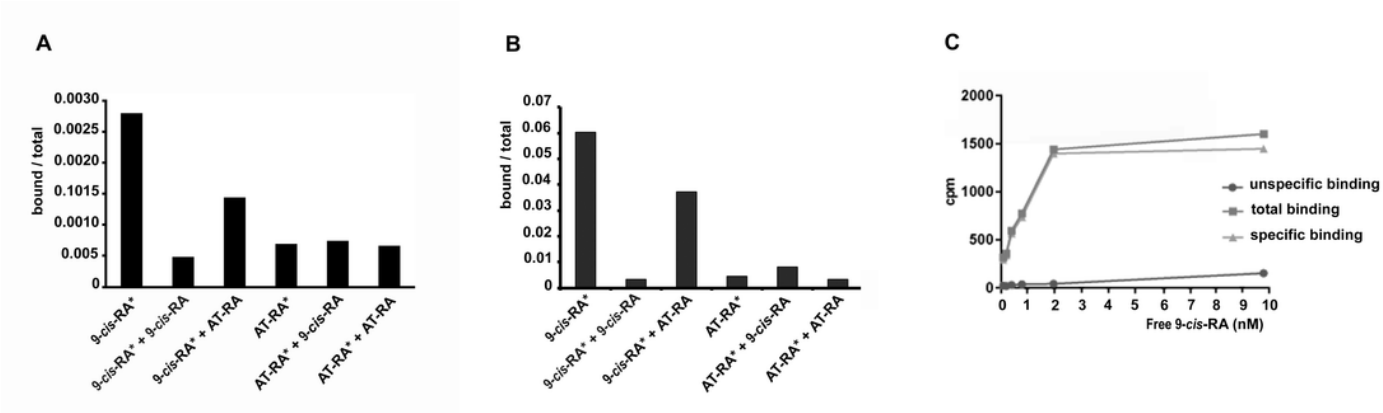

A
bound / total
0.0030
0.0025
0.0020
0.1015
0.0010
0.005
0
9-cis-RA*
9-cis-RA* + 9-cis-RA
9-cis-RA* + AT-RA
AT-RA*
AT-RA* + 9-cis-RA
AT-RA* + AT-RA
B
bound / total
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
9-cis-RA*
9-cis-RA* + 9-cis-RA
9-cis-RA* + AT-RA
AT-RA*
AT-RA* + 9-cis-RA
AT-RA* + AT-RA
C
cpm
2000
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10
Free 9-cis-RA (nM)
unspecific binding
total binding
specific binding

Figure 3

The effect of 9-*cis*-RA on the expression of the *T. adhaerens* closest putative homologue and likely orthologue of L-malate-NADP$^+$ oxidoreductase (EC1.1.1.40).

Ten to fifteen animals were cultured in the dark overnight with indicated ligands or in medium containing only the solvent used for ligand solutions. Total RNA and cDNA were prepared using identical conditions and diluted to the same working concentration suitable for ddPCR. In repeated experiments, incubation with 3 nM 9-*cis*-RA induced expression of the putative *T. adhaerens* L-malate-NADP$^+$ oxidoreductase more than 4 times. Incubation with 30 nM 9-*cis*-RA induced enzyme expression also, but to a lesser extent and 30 nM all-*trans*-RA (AT-RA) did not upregulate the expression of the predicted L-malate-NADP$^+$ oxidoreductase.

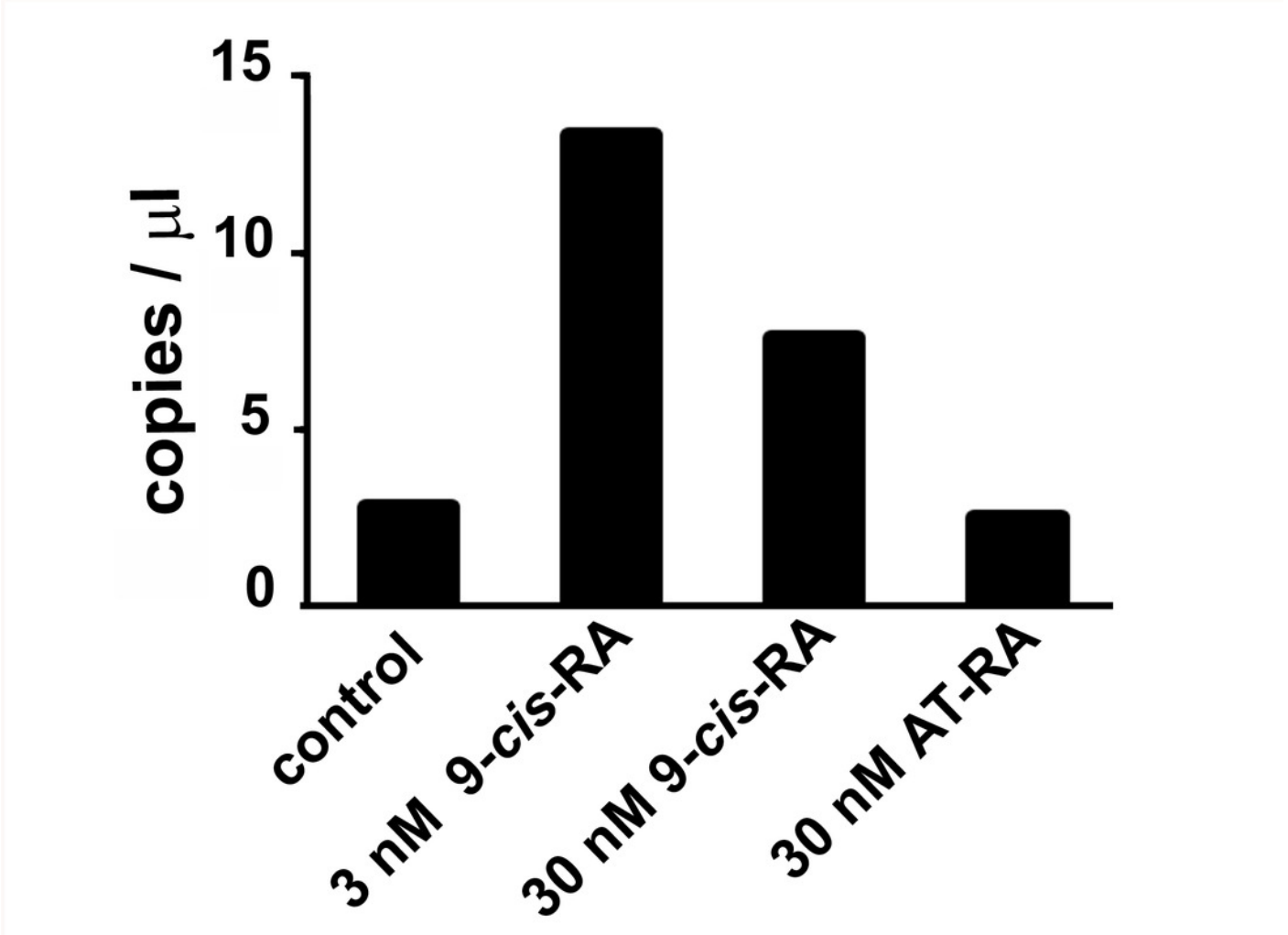


Figure 4

The effect of *9-cis*-RA on the expression of *T adhaerens* NRs.

A representative experiment of the expression of *T. adhaerens* NRs in animals exposed to various concentrations of 9-*cis*-RA expressed as a ratio of obtained values compared to the control using ddPCR. One and 3 nM 9-*cis*-RA upregulate RXR and ERR, but downregulate COUP-TF. The expression of *T. adhaerens* HNF4 is not affected by 9-*cis*-RA. The effect of the exposure to 9-*cis*-RA is stronger in the case of 1 nM 9-*cis*-RA compared to 3 nM 9-*cis*-RA. The exposure to 30 nM, as well as 50 nM concentrations of 9-*cis*-RA reverse the effect of 9-*cis*-RA on the expression of RXR and COUP-TF, but do not influence the expression of ERR. The level of expression of HNF4 is not changed by exposure of *T. adhaerens* to various concentrations of 9-*cis*-RA. The data suggest that a network sensitive to nanomolar concentrations of 9-*cis*-RA at the expressional level is formed by RXR, COUP-TF and ERR. All four NRs have conserved P-box (regions responsible for binding to response elements (RE) in gene promoters) and are likely to bind overlapping REs and to form a functional network.

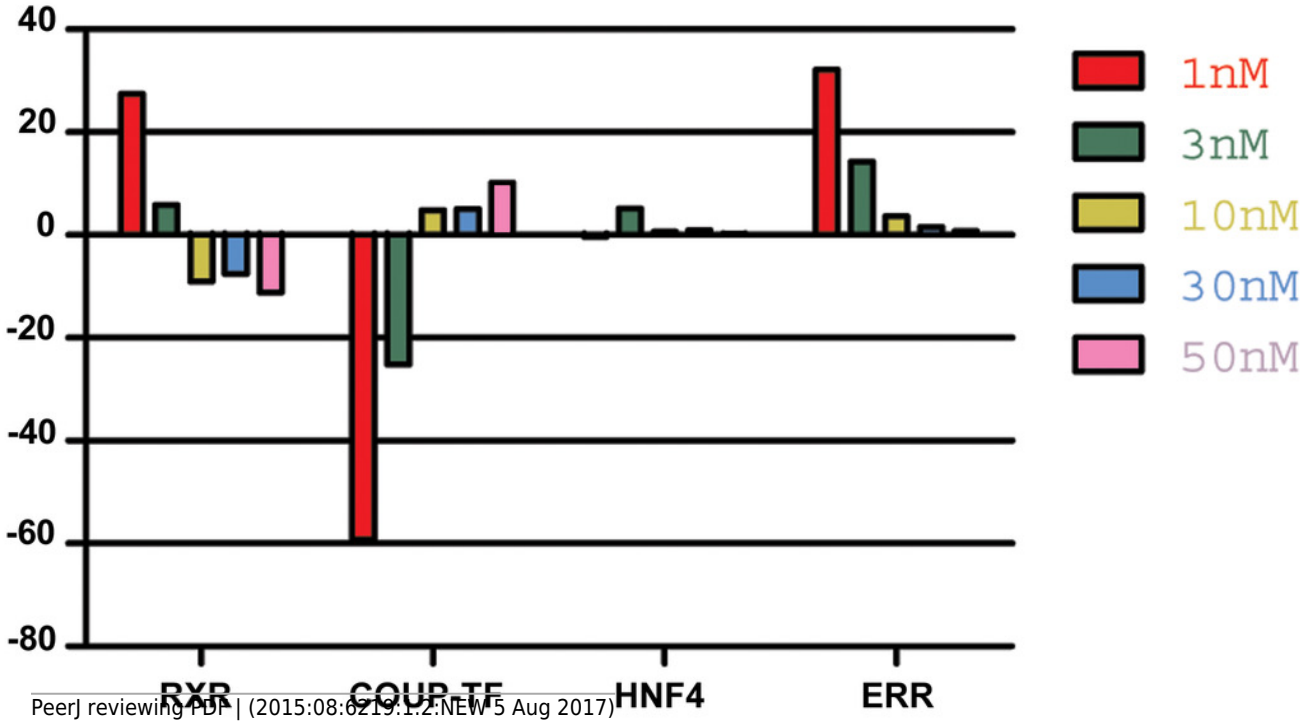


Figure 5

Phenotypes of *T. adhaerens* change at various feeding conditions.

T. adhaerens acquires various body shapes in individual cultures dependent on food availability and composition. At conditions maintained in stable and biologically equilibrated stock aquariums, *T. adhaerens* is usually small and pale with diameter varying from 50 µm to 400 µm while cultures with added algae contain large flat animals with diameter reaching up to 1 mm (A and B). In some cultures, animals grow as long stretching structures, reaching a length exceeding one or even several centimeters (C). The algal food makes the animals greenish, reddish, rusty or brown with variable proportion of prominent dark cells. Animal shapes also vary from flat and round with smooth circumference, to curved or ruffled circumference or animals with long projections. Bars represent 1mm in A, B, I, 1cm in C, 250 µm in D, 500 µm in E and H, 200 µm in F, and 100 µm in G.

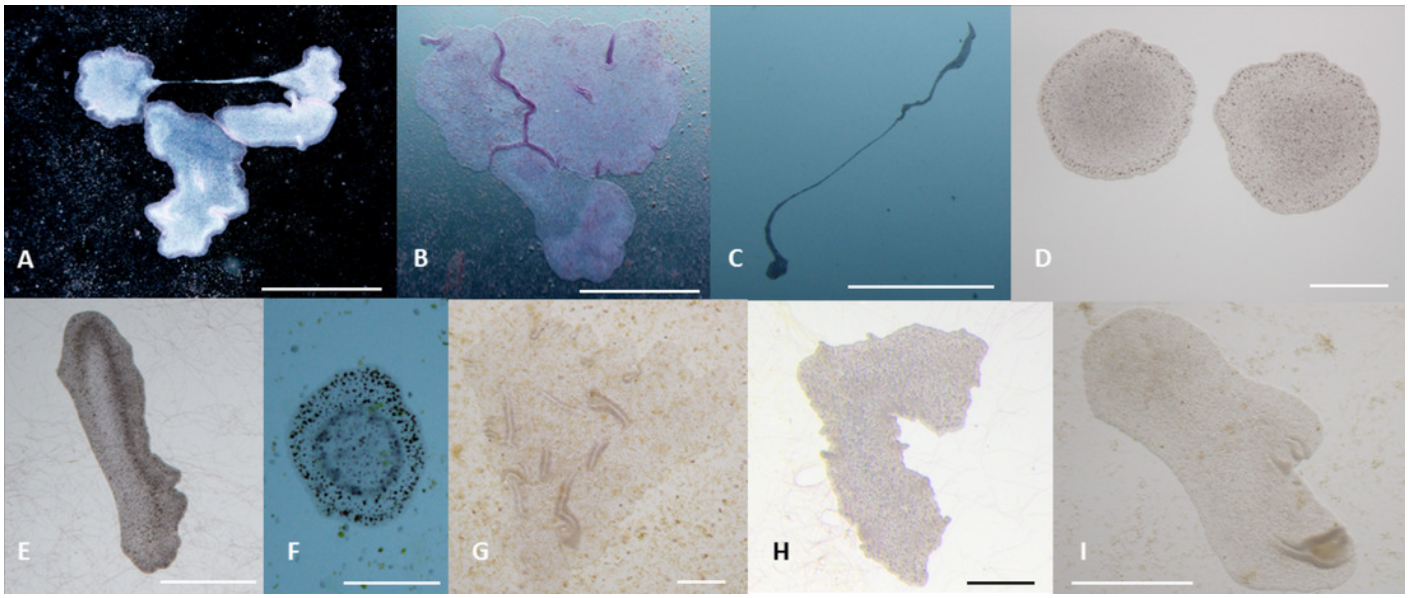


Figure 6(on next page)

Propagation of *T. adhaerens* depends on algal food composition.

Three cultures of 5 large animals in each were established and fed with the same number of algal cells consisting of *Chlorella sp.*, *Chlorella sp.* and *Rhodomonas salina* and *Chlorella sp.*, *Rhodomonas salina* and *Porphyridium cruentum*. While the culture fed with *Chlorella sp.* only doubled in the number of animals within a period of one week, cultures with red pigment containing algae multiplied more than 5 times and 10 times within the same time period.

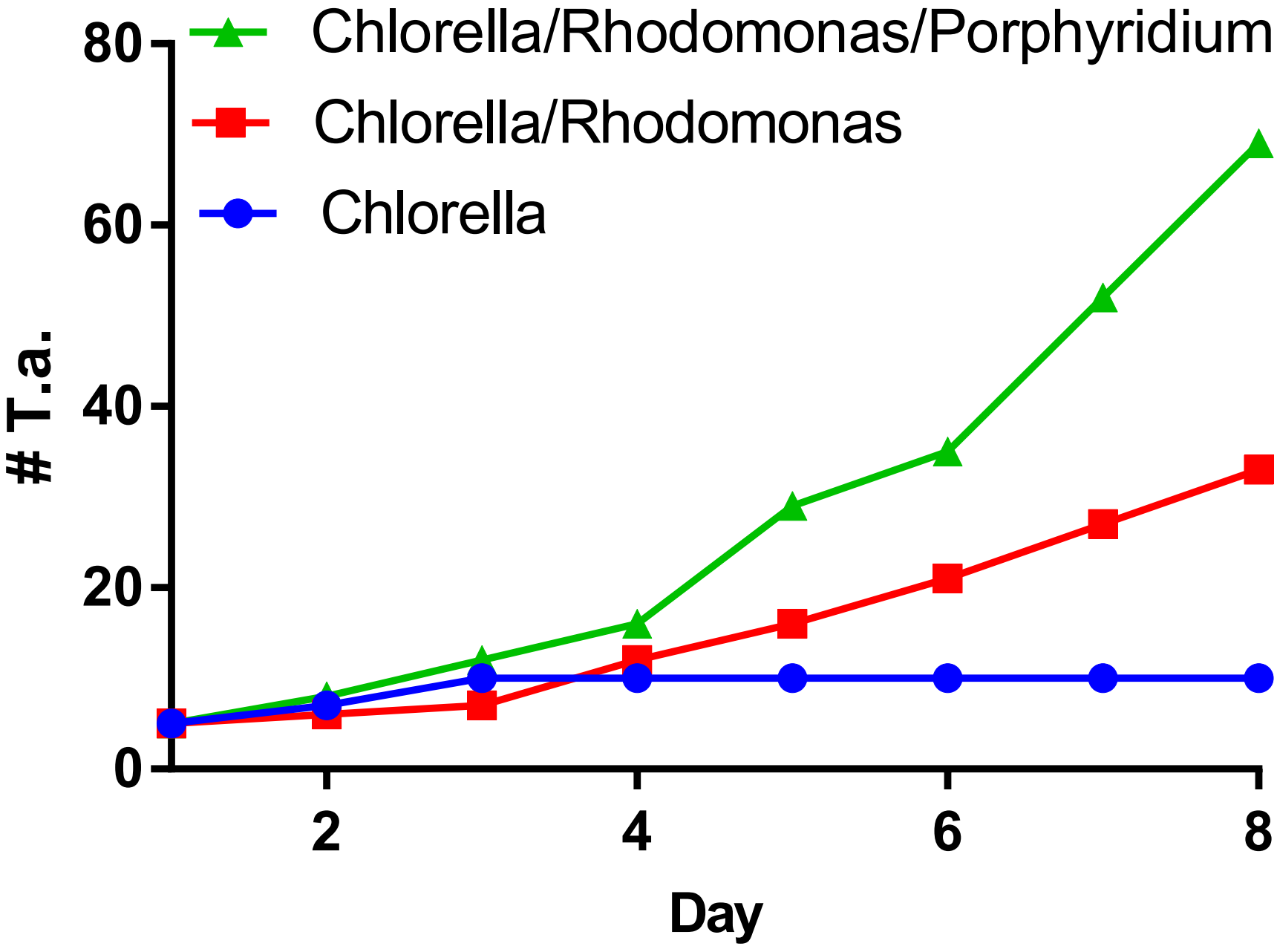

Chlorella/Rhodomonas/Porphyridium
Chlorella/Rhodomonas
Chlorella
T.a.
80
60
40
20
0
2
4
6
8
Day

Figure 7

The effect of algal food composition on *T. adhaerens* growth and appearance.

T. adhaerens was cultured similarly as shown in **Fig. 6** and all animals were photographed and analyzed using ImageJ program for their number (panel A), mean area (panel B), mean perimeter (panel C), mean perimeter/area ratio (panel D) and mean isometric quotient (panel E) after one week. Ch – stands for feeding with *Chlorella sp.*, Rh – *Rhodomonas salina*, Po – *Porphyridium cruentum*, and their combinations. 3Rh stands for a culture with three times higher concentration of *Rhodomonas salina* and 3Ch for three times higher concentration of *Chlorella sp.* Panel A shows that addition of *Rhodomonas salina* (Ch+Rh) greatly increases the number of animals observed after one week of culture. This effect is even more pronounced in cultures containing all three algae, while three times bigger concentration of only one type of algae (Ch and Rh) has little or no effect. This is even more pronounced when the area and perimeter are determined (panels B and C). Determination of the isoperimetric quotient in individual cultures indicates that cultures with *Rhodomonas salina* have a significantly smaller ratio, suggesting higher proliferative rate of structures at the animal circumference (panel E). Bars represent 95% confidence interval. Raw data are provided as Supplementary File S5 and S6.

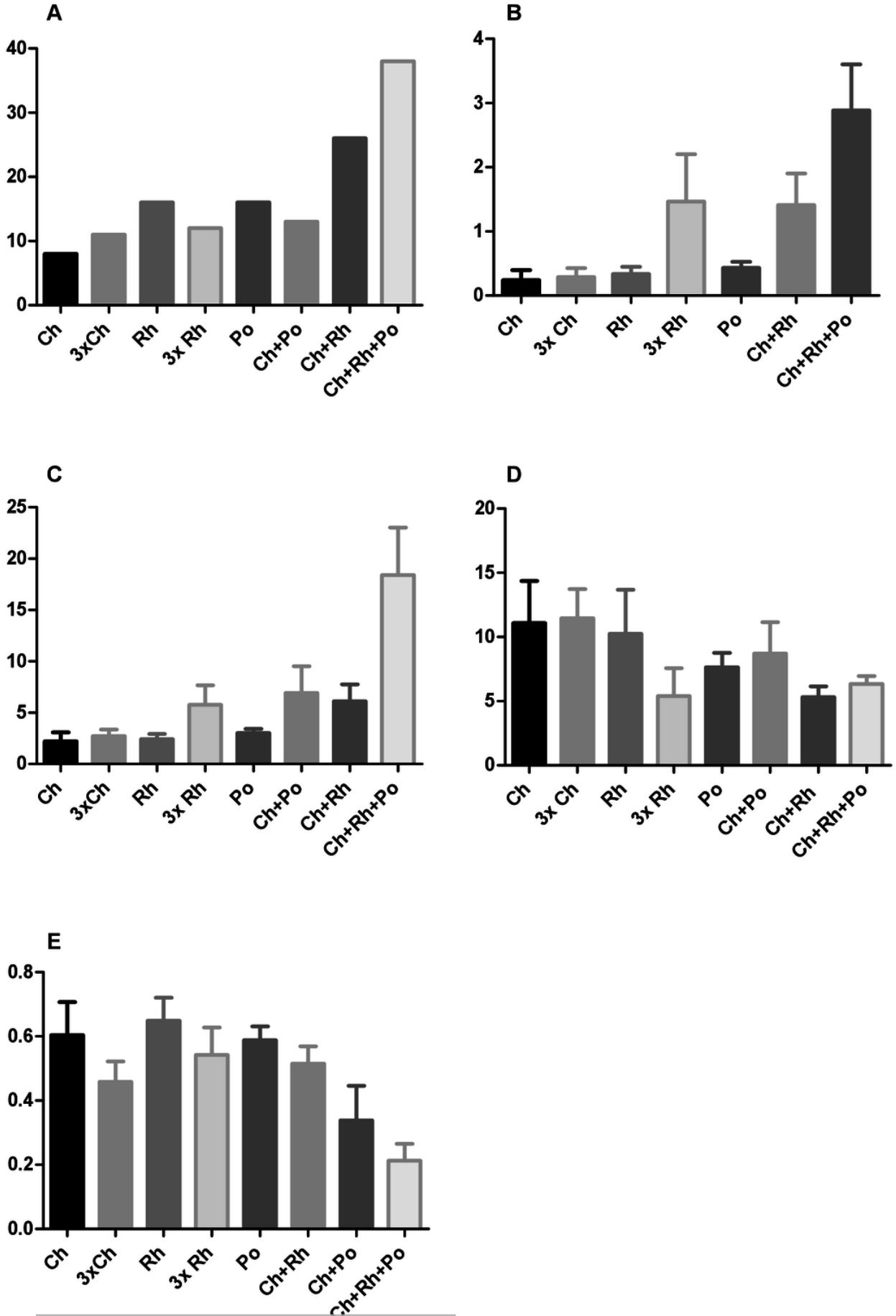
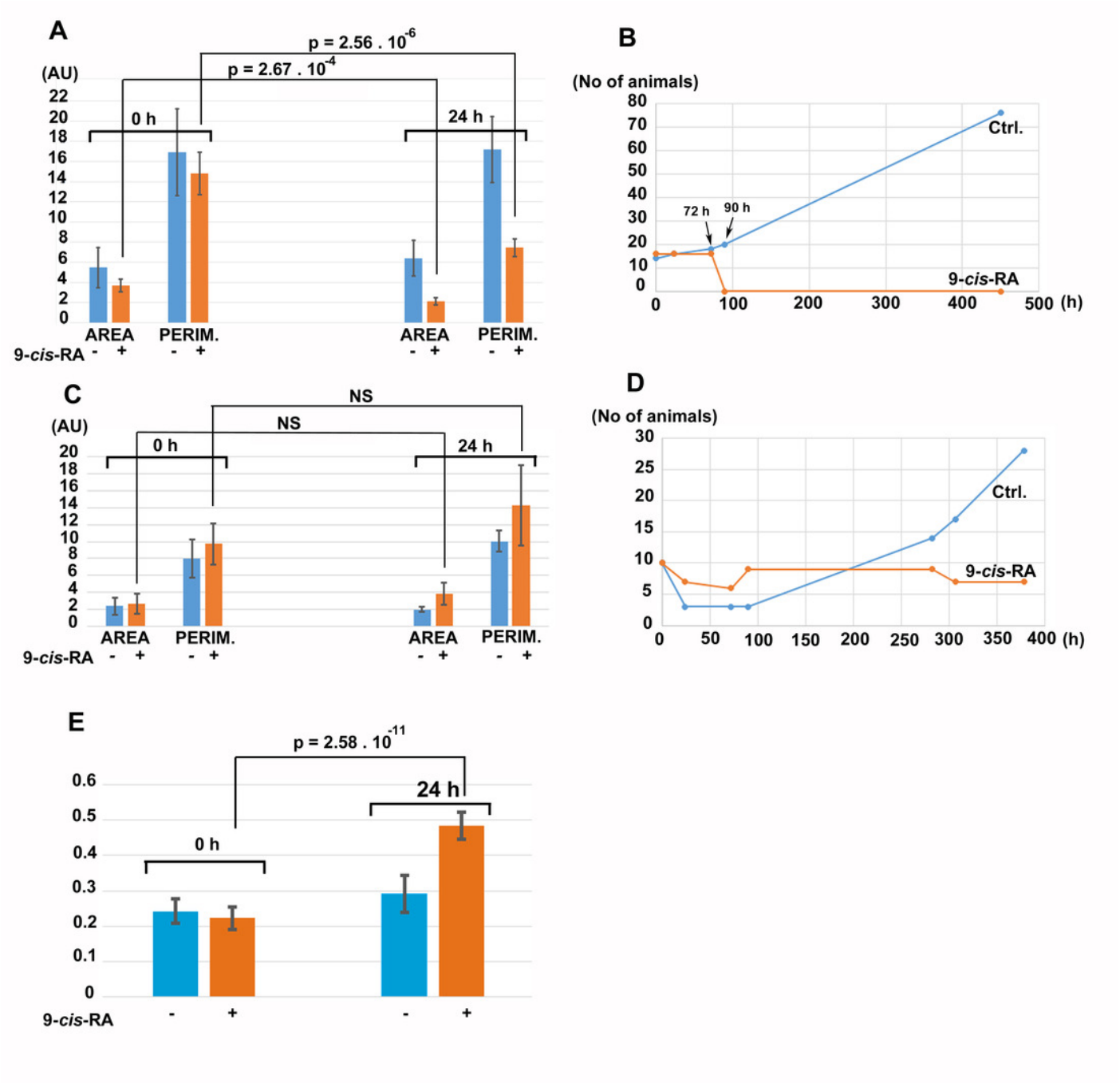

A
40
30
20
10
0
Ch
3xCh
Rh
3x Rh
Po
Ch+Po
Ch+Rh
Ch+Rh+Po
B
4
3
2
1
0
Ch
3x Ch
Rh
3x Rh
Po
Ch+Rh
Ch+Rh+Po
C
25
20
15
10
5
0
Ch
3xCh
Rh
3x Rh
Po
Ch+Po
Ch+Rh
Ch+Rh+Po
D
20
15
10
5
0
Ch
3x Ch
Rh
3x Rh
Po
Ch+Po
Ch+Rh
Ch+Rh+Po
E
0.8
0.6
0.4
0.2
0.0
Ch
3xCh
Rh
3x Rh
Po
Ch+Rh
Ch+Po
Ch+Rh+Po

Figure 8

The effect of 9-*cis*-RA on growth of *T. adhaerens* fed by *Porphyridium cruentum* or *Chlorella sp.*

Panel A shows the comparison of the total area and total perimeter of control *T. adhaerens* and *T. adhaerens* treated by 9-*cis*-RA for 24 h expressed as arbitrary units derived from pixel measurements obtained at 24 h and compared to values obtained immediately prior to incubation. The data indicates that animals incubated in 3.3 nM 9-*cis*-RA decreased their area and perimeter to approximately 50 % in comparison to control animals. Panel B shows the development of cultures over a three-week period. The animals treated by 9-*cis*-RA developed a balloon-like phenotype at 72 h of incubation and died at 90 h of incubation. Panels C and D show the data obtained with *T. adhaerens* originating from cultures fed by naturally established biofilms in laboraotory aquariums, fed by *Chlorella sp*. and treated similarly as shown in panel A and B. Panel E shows the analysis of circularity of animals presented in panel A and documents that animals treated by 9-*cis*-RA increased their circularity already at 24 h of exposure suggesting arrest of growth of animal peripheral tissues.


A
p = 2.56 . 10-6
p = 2.67 . 10-4
(AU)
0 h
24 h
AREA
PERIM.
9-cis-RA
B
(No of animals)
Ctrl.
72 h
90 h
9-cis-RA
(h)
C
NS
NS
(AU)
0 h
24 h
AREA
PERIM.
9-cis-RA
D
(No of animals)
Ctrl.
9-cis-RA
(h)
E
p = 2.58 . 10-11
0 h
24 h
9-cis-RA