A peer-reviewed version of this preprint was published in PeerJ on 29 September 2017.

Novotný JP, Chughtai AA, Kostrouchová M, Kostrouchová V, Kostrouch D, Kaššák F, Kaňa R, Schierwater B, Kostrouchová M, Kostrouch Z. 2017. *Trichoplax adhaerens* reveals a network of nuclear receptors sensitive to 9-*cis*-retinoic acid at the base of metazoan evolution. PeerJ 5:e3789 https://doi.org/10.7717/peerj.3789

Trichoplax adhaerens reveals an endocrine-like network sensitive to 9-*cis*-retinoic acid at the base of metazoan evolution

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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 homologues of RXR (NR2B), HNF4 (NR2A), COUP (NR2F) and ERR (NR3B) that show a high degree of similarity with human homologues. 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* homologue 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 profound effect on *T. adhaerens* growth and appearance. 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.

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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 homologues of RXR (NR2B), HNF4 (NR2A), COUP (NR2F) and ERR (NR3B) that show a high degree of similarity with human homologues. 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* homologue 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 profound effect on *T. adhaerens* growth and appearance. 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 prokaryotic 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 conserved 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 for 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). This is accompanied by two essential questions: to what degree is the plasticity of ligand selection is a fundamental property of NRs; and what might be the origin of specific ligand binding by NRs. It has been suggested that the original NR, which is the ancestral NR possessing gene regulatory capacity, may have been an unliganded molecular regulator. 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).

A search for NRs that may be closely related to an ancient ancestor of the NR family led to the discovery of an RXR homologue in Cnidaria (Kostrouch et al. 1998). Surprisingly, this receptor showed not only extremely high degree of sequence homology 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 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 homologue 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. Genome analysis of the basal metazoan *Trichoplax adhaerens* by whole genome sequencing revealed four highly conserved nuclear receptors, namely homologues of HNF4 (NR2A), RXR (NR2B), ERR (NR3) and COUP (NR2F) (Srivastava et al. 2008). 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 homologues is high, as it is in the case of the cubomedusan RXR. *T. adhaerens,* which shows characteristics of a basal

metazoan with only 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 homologue functionally. Our results show that *T. adhaerens* RXR binds 9-*cis*-RA with an affinity comparable to that of vertebrate and jellyfish RXRs and *T. adhaerens* responds to nanomolar concentrations of 9-*cis*-RA with a transcriptional upregulation of the putative homologue of 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 in 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.

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

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

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

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

Each PCR product was then ligated into pCR®2.1-TOPO® or pCR®4-TOPO® vector with the classic TA Cloning Kit and TOPO TA Cloning Kit (Invitrogen™), respectively. The ligated products were then transformed using One Shot® TOP10 Chemically Competent *E. coli* and cultured on LB Agar plates containing 100 µg/ml ampicillin. Plasmid DNA was extracted from obtained colonies and screened for mutations by sequencing using vector specific M13 forward and reverse primers. Only non-mutated sequences were used in subsequent experiments.

155 The RXR fragments were then restricted and inserted into pGEX-2T vector system for bacterial
156 expression (Addgene, Cambridge, MA, USA). Proper insertion was verified by sequencing.
157

158 ## Protein expression

159 BL21 pLysS bacteria were transformed with previously described RXR mRNA inserted
160 into pGEX-2T vector. Stocks of transformed bacteria were stored in 8% glycerol according to the
161 Novagen pET System Manual (11th edition)
162 (https://www.google.cz/search?q=Novagen+pET+System+Manual+&ie=utf-8&oe=utf-
163 8&client=firefox-b&gfe_rd=cr&ei=T9z1WMHJDsni8AfpmoGoCQ). For protein expression,
164 bacteria were scraped from stock and incubated in Liquid Broth (LB) with ampicillin (100 µg/ml)
165 and chloramphenicol (34 µg/ml) overnight. The culture was then used to inoculate 100 ml of LB
166 + antibiotics and grown to OD600 = 0.6-0.8 at 37 ºC, then induced with 100 µl 1M IPTG
167 (isopropyl-D-thiogalactopyranoside) (Sigma-Aldrich, St. Louis, MO, USA). and moved to 25 ºC
168 (RT) for 5 h. The culture was then spun at 9000 xg for 15 min and the supernatant discarded. The
169 bacterial pellet was resuspended in 10 ml GST binding buffer (25 mM Tris pH 7.5, 150 mM NaCl,
170 1 mM EDTA + protease inhibitor (S8820 Sigma Fast, Sigma-Aldrich, St. Louis, MO, USA or
171 cOmplete™, EDTA-free Protease Inhibitor Cocktail, Roche, Basel, Switzerland)). Bacteria were
172 then lysed by 6 x 20 s ultrasonication on ice (50 watts, 30kHz, highest setting – 100%) (Ultrasonic
173 Processor UP50H, (Hielscher Ultrasonics GmbH, Teltow, Germany) and subsequently incubated
174 with 15-20 mg glutathione agarose beads (Sigma-Aldrich®) prepared according to manufacturer's
175 instructions. Incubation took place at 4 ºC for about 10 h and beads were washed according to
176 instructions, resuspended in regeneration buffer (50mM Tris-HCl pH7.4, 1mM EDTA, 120 mM
177 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.

The size of the GST-RXR fusion protein was checked by SDS-PAGE. Thrombin cleavage was performed at RT for 4 h.

Radioactive *9-cis-RA* binding assay

Radioactive 9-*cis*-RA and 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-TaRXR fusion protein on beads with about 375 ng/assay or thrombin-cleaved TaRXR. 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 centrifugation at 1300 xg 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 suspended in binding buffer (12.7 mg/100µl) was added to the assay and mixed twice, collecting the apatite slurry by centrifugation (15 s at 600 xg). 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 60W, 120V yellow light bulb (BugLite, General Electric Co, Nela Parc, Cleveland Oh, USA) as described (Cahnmann 1995).

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 lightbulb on a daylight background from late spring to mid-summer in the laboratory located at 50.0703122N, 14.4293456E 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.

Treatment of *T. adhaerens* with retinoic acids

Incubation of the animals was done overnight in the absence of light. Each batch within an experiment was derived from similar cultures and fed with similar amounts and composition of algae. All experiments were started in a dark room with indirect yellow light illumination (similarly as in case of the ligand binding studies) and further incubations were done in the dark.

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 were removed per 100µl TRIZol reagent. RNA purification was performed according to manufacturer's instructions. Total RNA was measured by a UV spectrophotometer and used as a reference for normalization.

Reverse transcription was performed with SuperScript III Reverse Transcriptase according to manufacturer's instructions. The cDNA was then mixed with ddPCR Supermix (Bio-Rad) according to the manufacturer's instructions (corresponding to 100-500 ng of RNA) 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 (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

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

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

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

Identification of *T. adhaerens* homologue of L-malate-NADP+ oxidoreductase (EC 1.1.1.40)

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

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 reference we screened the *Trichoplax* JGI database for RXR homologues 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 RXRalpha.

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 were present (**Fig. 1**) and also amino acid residues shown as critical for binding 9-*cis*-retinoic acid (Egea et al. 2000) . Out of 11 amino acid residues 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 (A271, A272, Q 275, L 309, F 313, R 316, L 326, A 327, R 371, C 432, H 435), 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 **(Fig 2).**

The remaining three NRs identified in the *T. adhaerens* genome show also very high overall sequence identity with vertebrate homologues **(Table 1).** Alignments of *T. adhaerens* HNF4, COUP and ERR with homologues from selected species can be found in **Supplementary File S2**.

Table 1. BLASTP comparison of *T. adhaerens* NRs with the human closest homologues and cross-species conservation of RXR homologues

T. adhaerens homologue	Percent identity (*H. sapiens* homologue)
TaRXR	66% (RXRα)
TaCOUP	43% (COUP1)
TaHNF4	67% (HNF4α)
TaERR	46% (ERR3)

Drosophila melanogaster homologue	Percent identity (*H. sapiens* homologue)
USP	47% (RXRα)

T. adhaerens homologue	Cnidaria (*T. cysotophora* homologue)
TaRXR	35% (jRXR)

Cnidaria (*T. cysotophora* homologue)	Percent identity (*H. sapiens* homologue)
jRXR	48% (RXRα)

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

Consistent with the bioinformatically high conservation of the LBD shown, we were able to show that TaRXR binds 9-*cis*-retinoic acid with high affinity and specificity (**Fig. 3A**). A 9-*cis*-RA binding assay to determine the dissociation constant of TaRXR indicated high affinity binding with a saturation plateau from 5 nM to 10 nM. This was clearly observed in repeated experiments (**Fig. 3B**). In contrast, TaRXR does not show high affinity displaceable binding of all-*trans*-retinoic acid. GST-RXR and Thrombin-cleaved RXR showed similar 9-*cis*-RA binding capability (not shown).

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 malic enzyme gene after incubation of *T. adhaerens* with 9-*cis*-RA, but not with all-*trans*-RA **(Fig 4)**. 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.

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

From the experience we gained by culturing *T. adhaerens*, as well as from the previous experiments we knew that the culture conditions can dramatically influence phenotype. Having

the possible developmental functions of the ancestral NRs in mind, we raised the question whether the expression patterns of the NRs reflect changes in phenotype.

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

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

Analysis of NR expression pattern in animals incubated with different concentrations of 9-*cis*-RA, revealed a relative increase in RXR expression at low nanomolar concentrations (<10 nM) in repeated experiments. In contrast, further increase of 9-*cis*-RA resulted in smaller changes compared to the expression pattern of NRs in control animals or even reverted the values observed in low nanomolar conditions (**Fig 5**).

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

371 *T. adhaerens* retrieved from laboratory aquariums used for the stock cultures were
372 relatively similar in appearance and included small round animals containing approximately 50
373 cells and grew to animals with an approximate diameter of 0.2 mm and rarely were bigger. Their
374 rate of multiplication when transferred to Petri dishes was doubling in one month or even one
375 week, depending on whether the glass was covered by microbial and algal films established during
376 culturing in aquariums. We attempted to use several defined algae as artificial food. They included
377 *Pyrrenomonas helgolandii*, *Picocystis salinarium*, *Tetraselmis subcoriformis*, *Rhodomonas*
378 *salina*, *Phaeodactylum tricornutum*, *Porphyridium cruentum* and *Chlorella sp*. Individual
379 subcultures of *T. adhaerens* differed in the rate of propagation and appearance as well as colors
380 that were varying from greenish to brown and reddish taints depending on the food that was used
381 as singular species food or mixtures (**Fig 6**). Also, contaminants from the original algal food, which
382 prevailed in some cultures, had an influence on *T. adhaerens* growth and behavior. In controlled
383 experiments, it became clear that some food components or food compositions are more affecting
384 the growth and appearance of *T. adherens* than food availability. When *T. adhaerens* were fed
385 with equal amounts of algal cells (although they differed in size and expected digestibility), the
386 addition of algae containing red pigments - Cryptophytes (*Pyrrenomonas helgolandii* and
387 *Rhodomonas salina*) or Rhodophyta (*Porphyridium cruentum*) - had a strong positive effect on *T.*
388 *adhaerens* growth, especially in combination with the green algae *Chlorella sp.* (**Fig 7**).
389 Furthermore, the addition of *Porphyridium cruentum* to *Chlorella sp.* resulted in significant
390 change in circularity, while feeding *T. adhaerens* with triple food containing *Chlorella*,
391 *Rhodomonas* and *Porphyridium* showed the most pronounced effect. Culturing *T. adhaerens* on
392 either single food showed similar isoperimetric values (**Fig 8).**
393

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

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

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

The four NRs found in the genome of *T. adhaerens* are relatively closely related to their vertebrate counterparts, RXR (NR2B), HNF4 (NR2A), COUP (NR2F) and ERR (NR3) (Srivastava et al. 2008). Among them, *T. adhaerens* RXR and HNF4 show the highest degree of identity in protein sequence and the relatedness of *T. adhaerens* RXR (TaRXR) to human RXR is similar to that of *Tripedalia cystophora* RXR (jRXR) (Kostrouch et al. 1998), which has also been shown to bind 9cis-RA at nanomolar concentrations. These results suggest that TaRXR is structurally and also functionally very closely related to its vertebrate counterparts, most probably representing the most basal liganded NR of all Metazoa known today.

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

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

9-*cis*-RA affects the expression of a *T. adhaerens* homologue of a conserved metabolically active enzyme, L-malate-NADP+ oxidoreductase

A biological role of 9-*cis*-RA binding with high affinity to the placozoan RXR receptor is supported by our *in vivo* experiments. In search for genes that may be under the regulation of

TaRXR, we identified a putative homologue of vertebrate L-malate-NADP+ oxidoreductase (EC 1.1.1.40) and analyzed the effect of 9-*cis*-RA or all-*trans*-RA on its expression. In agreement with our binding experiments, we observed induction of this enzyme's expression at low nanomolar concentrations of 9-cis-RA (1 to 10 nM). Interestingly, higher concentrations of 9-*cis*-RA (30 nM) had a smaller effect on expression and all-*trans*-RA had no effect up to 30 nM concentrations. A plausible explanation for this could be that 9-*cis*-RA also acts as a ligand for other *T. adhaerens* NRs which may have an opposite effect on the expression of L-malate-NADP+ oxidoreductase. Furthermore, 9-*cis*-RA could act as a 'supranatural' ligand and the continuous occupation of TaRXR by this high affinity ligand may interfere with the normal function of the receptor which has been demonstrated in the case of the glucocorticoid receptor (Schaaf & Cidlowski 2003; George et al. 2009).

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

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

Since autoregulation and cross-regulation of NRs by their specific ligands is well documented for a large number of nuclear receptors (Tata 1994), we searched if 9-*cis*-RA affects the expression of TaRXR mRNA relative to the other *T. adhaerens* NRs. Our *in vivo* experiments showed not only effects on specific gene expression in response to very low concentrations of 9-cis-RA (at 1 or 3 nM), but also an additional dose-dependent reverse effect of higher concentrations. This is likely to be in line with our binding experiments that suggested the possibility of an additional binding site or sites with higher capacity and lower affinity. We also cannot rule out that higher concentrations of 9-*cis*-RA affect some of the three remaining *T. adhaerens* NRs. Nevertheless, an inhibitory effect of 9-*cis*-RA on the expression of its cognate receptor at the protein level (through protein degradation) was reported (Nomura et al. 1999).

Although it is not clear if 9-*cis*-RA is the natural ligand for RXRs (Wolf 2006; Ruhl et al. 2015) conserved in all metazoan phyla studied to date, we show not only that 9-*cis*-RA binds TaRXR with nanomolar affinity but also positively regulates its expression, which resembles auto-activation of several NRs in vertebrates [e.g. ER and TR (Tata 1994; Bagamasbad & Denver 2011)]. Furthermore, three out of four NRs constituting the NR complement in *T. adhaerens* respond to treatment by 9-*cis*-RA at transcriptional level. Two NRs, RXR itself and ERR respond positively to nanomolar concentrations of 9-*cis*-RA, while COUP, which often acts as an inhibitor of specific gene expression (Tran et al. 1992), is regulated negatively by 9-*cis*-RA. COUP was recently shown to be inactivated by small hydrophobic molecules (Le Guevel et al. 2017). The regulatory connections of *T. adhaerens* NRs places the auto-regulation and cross-regulation of NRs to the base of metazoan evolution.

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

484 At first glance, *T. adhaerens* seems to benefit from any source of biological material on
485 surfaces that can be digested and absorbed by its digestive system (e.g. aquarium microorganisms
486 and detritus). Feeding with certain live microorganisms in laboratory cultures, however,
487 dramatically changes the dynamics of *T. adhaerens* cultures, such as shape, size, color, body
488 transparency, growth and divisions of the animal. For example, we observed poor growth and
489 reproduction rates of *T. adhaerens* fed solely on *Chlorella sp.* even at a relatively high density. In
490 contrast, cultures fed with red pigment containing *Rhodomonas salina* showed much faster
491 proliferation and led, in part, to the formation of giant animals, seeming to halt their division.
492 Despite *Porphyridium cruentum* containing similar pigments as *Rhodomonas*, such as
493 phycoerythrin, cultures grown with *Porphyridium* as the main nutrient source did not show
494 phenotypical abnormalities but addition of it to a culture with *Chlorella* and *Rhodomonas* resulted
495 in an additive effect on reproduction rate.

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

503 Our experiments provide evidence that food composition is more important for *T.*
504 *adhaerens* growth and propagation than its quantity, which is in line with the recent finding of
505 phosphate and nitrate playing important roles determining distribution of placozoans around the
506 globe (Paknia & Schierwater 2015). It indicates that food constituents, especially those present in

the algae containing phycobilin based red pigments like *Rhodomonas salina* and *Porphyridium cruentum* might possess hormone-like molecules or molecules resulting in hormone-like metabolites in *T. adhaerens* that act through the NR complement and, indeed, analysis of NRs in differently sized animals indicates impact of food composition on NR expression.

When viewed together, our work shows that the presence of 9-*cis*-RA binding RXR in Placozoa argues for the existence of ligand regulated NRs at the base of metazoan evolution. This work also suggests that an endocrine-like network was present in early metazoans, likely to be an ancestral regulatory network linking nutritional or environmental factors with the regulation of gene expression that is mediated by NRs. 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 regulatory pathways (King et al. 2008). On the other hand in fungi, the sister group of Holozoa, (Shalchian-Tabrizi et al. 2008) transcription factors surprisingly similar to metazoan NRs evolved independently possibly for the regulation of metabolism and response to xenobiotics (Thakur et al. 2008; Naar & Thakur 2009). Thus, the evolution of NRs seems to be associated with two key evolutionary features of metazoans: multicellularity and heterotrophy.

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

In conclusion, the *T. adhaerens* endocrine-like network supports the hypothesis of a basic regulatory mechanism by NRs, which may have been subspecialized with the appearance of new NRs in order to cope with new environmental and behavioral challenges during the course of early metazoan evolution.

Acknowledgements

The authors thank NCBI for accessibility of data and bioinformatics support and Joint Genome Institute of United States Department of Energy (http://jgi.doe.gov/) (Nordberg et al. 2014).

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712

Figure 1(on next page)

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. 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/).

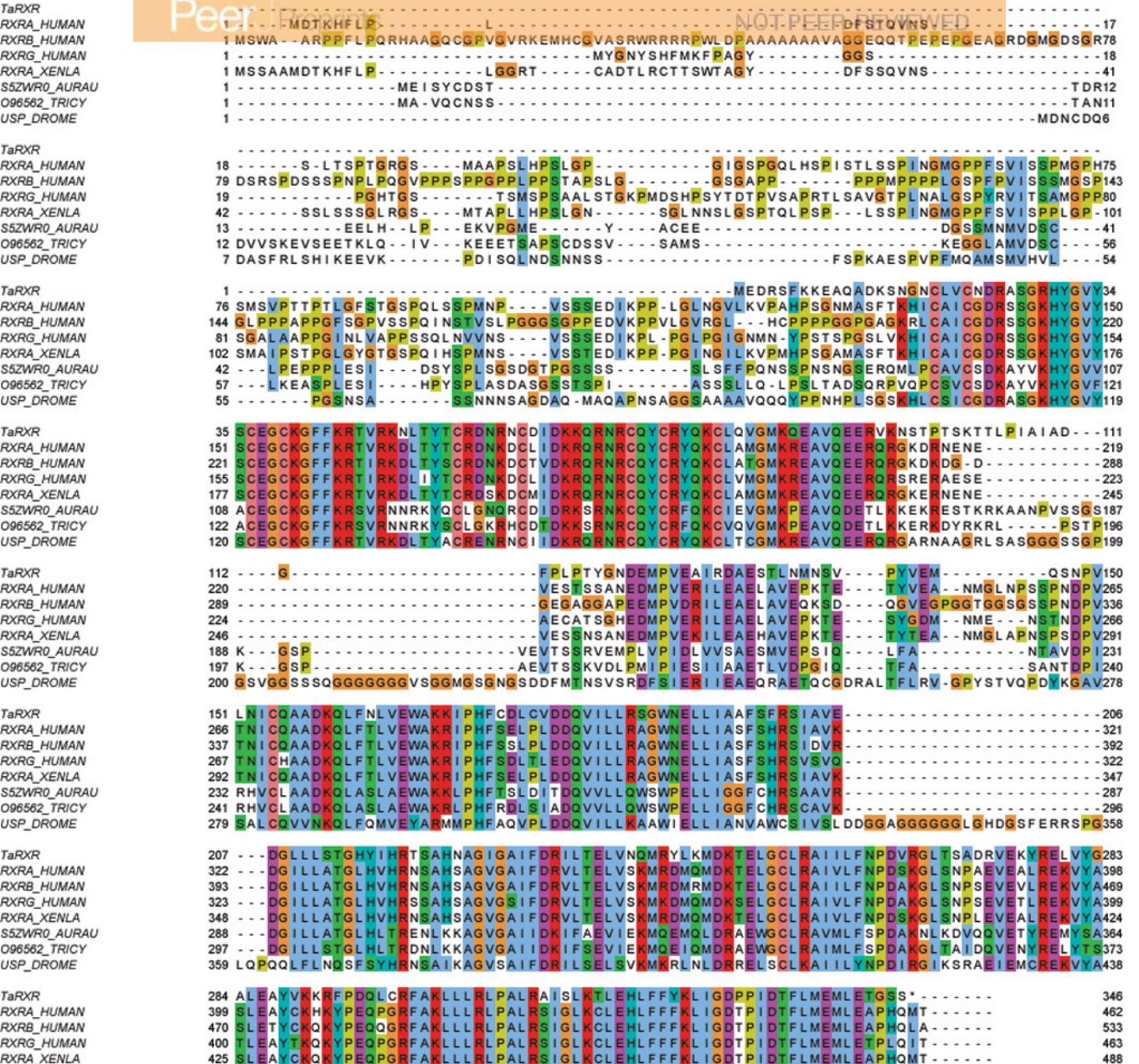

Figure 2

Phylogenic tree of sequences shown in Fig. 1.

Phylogenetic tree constructed with PhyML algorithm with a 100 bootstrap and SPR distance computation visualized by FigTree of selected metazoan RXR sequences shown in Fig. 1 (and listed in **Supplementary File S1**). Bootstrap values are shown next to nodes.

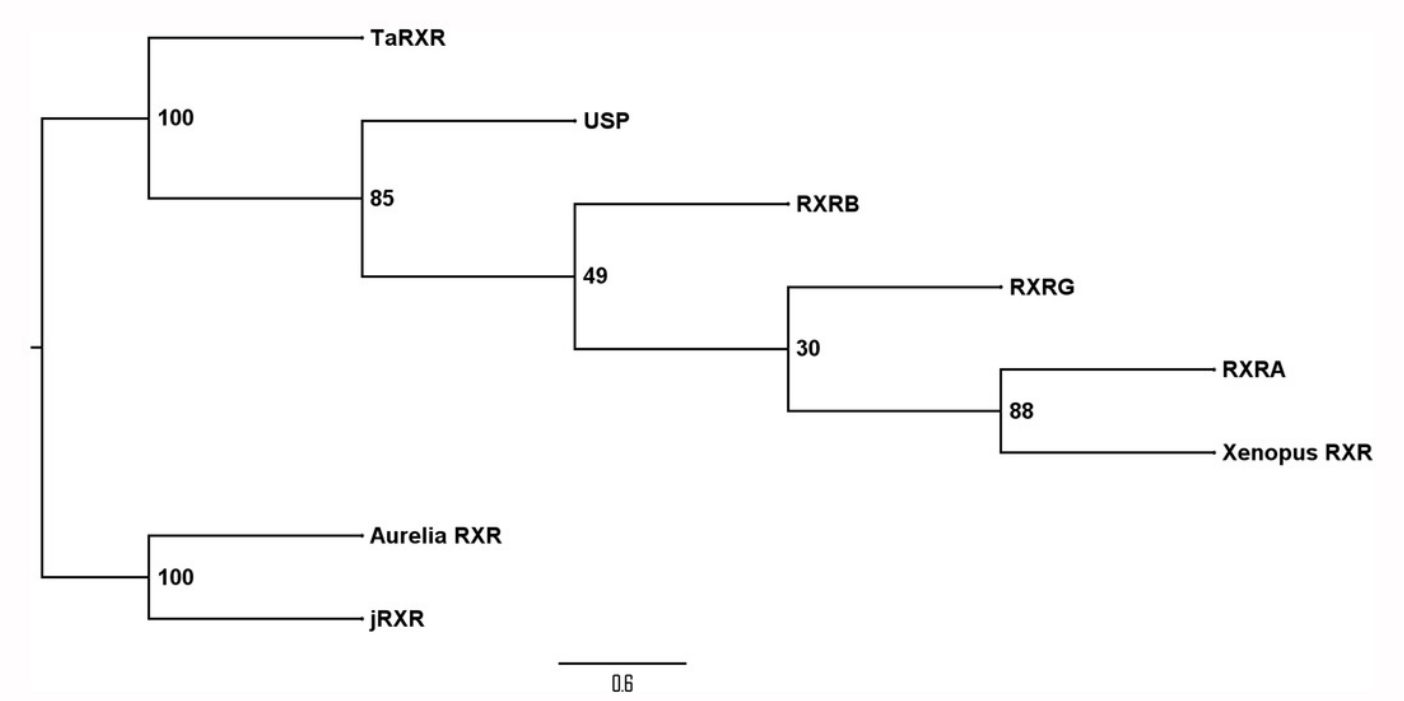

Figure 3

Binding of retinoic acids to TaRXR.

A – Single point analysis of binding preference of *T. adhaerens* RXR to 9-*cis*-RA over all-*trans*-RA. Radioactive 9-*cis*-RA binds at a concentration of 4 nM to 200 micrograms of *T. adhaerens* RXR (dark column). 200x excess of unlabeled *9-cis*-RA displaces more than 80 % labeled *9-cis*-RA from binding to *T. adhaerens* RXR (second column) while the same molar excess of all-*trans*-RA (marked as AT-RA) (which is likely to contain approximately 1% spontaneously isomerized *9-cis*- RA) competes away less than 50 % of bound labeled 9-*cis*-RA (column 3 from left). Radioactive all-*trans*-RA at identical conditions binds only slightly more than observed the non-specific binding with 9-*cis*-RA (column 4 from left) and this interaction is not displaced by the excess of non-labeled 9-*cis*-RA nor non-labeled all-*trans*-RA. B – Kinetic analysis of binding of 9-*cis*-RA to *T. adhaerens* RXR. The plateau is reached at around 3 to 5 x10^{-9} M.

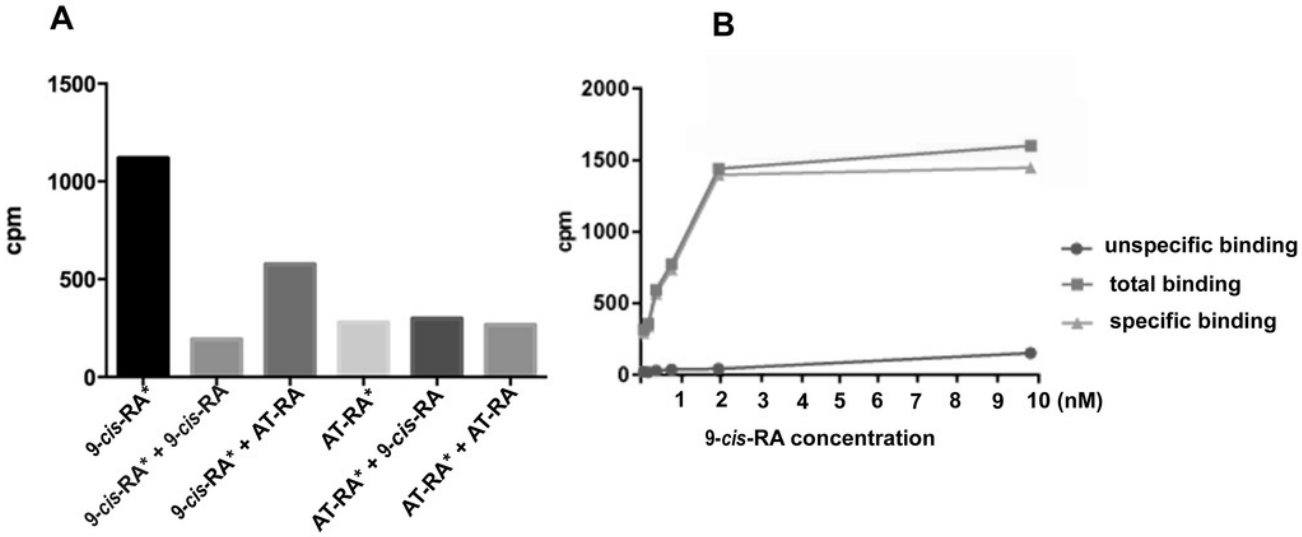

Figure 4

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 working concentrations 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.

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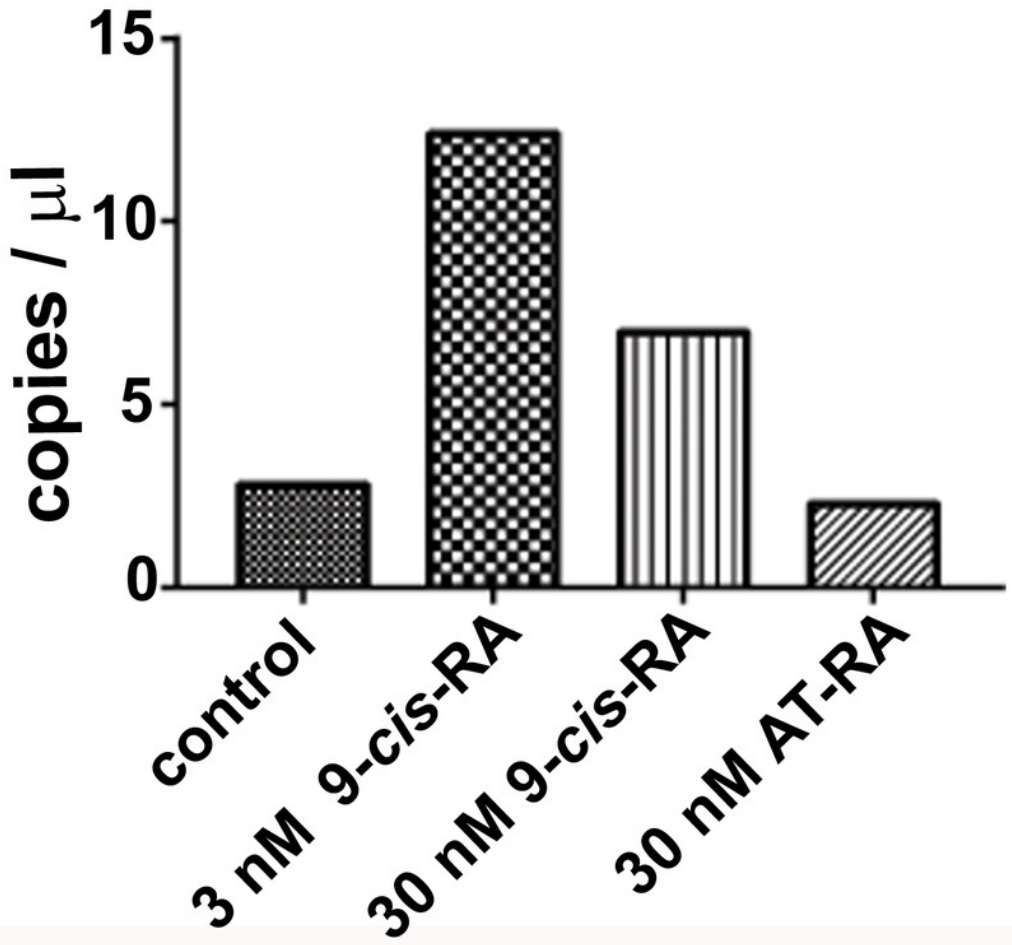

Figure 5

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 change of the ratio compared to the control. One and 3 nM 9-*cis*-RA upregulate RXR and ERR, while downregulating COUP. The expression of *T. adhaerens* HNF4 is not changed. Similarly, as in the case of the putative L-malate-NADP+ oxidoreductase homologue shown in **Fig 4**, the effect is stronger in the case of 1 nM 9-*cis*-RA compared to 3 nM, 30 nM, as well as 50 nM concentrations reverse the effect on RXR and COUP, but do not influence the expression of ERR. The data suggest that a network sensitive to nanomolar concentrations of 9-*cis*-RA at an expressional level is formed by RXR, COUP, HNF4 and ERR.

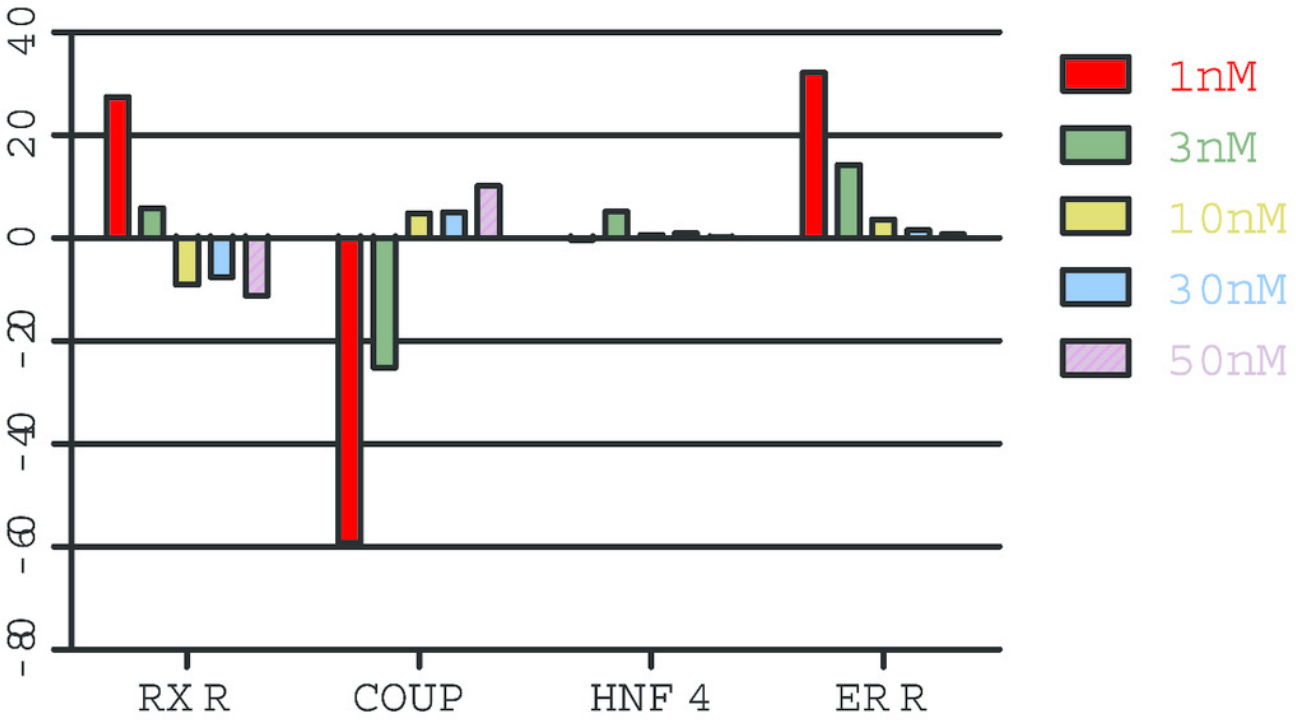

Figure 6

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.

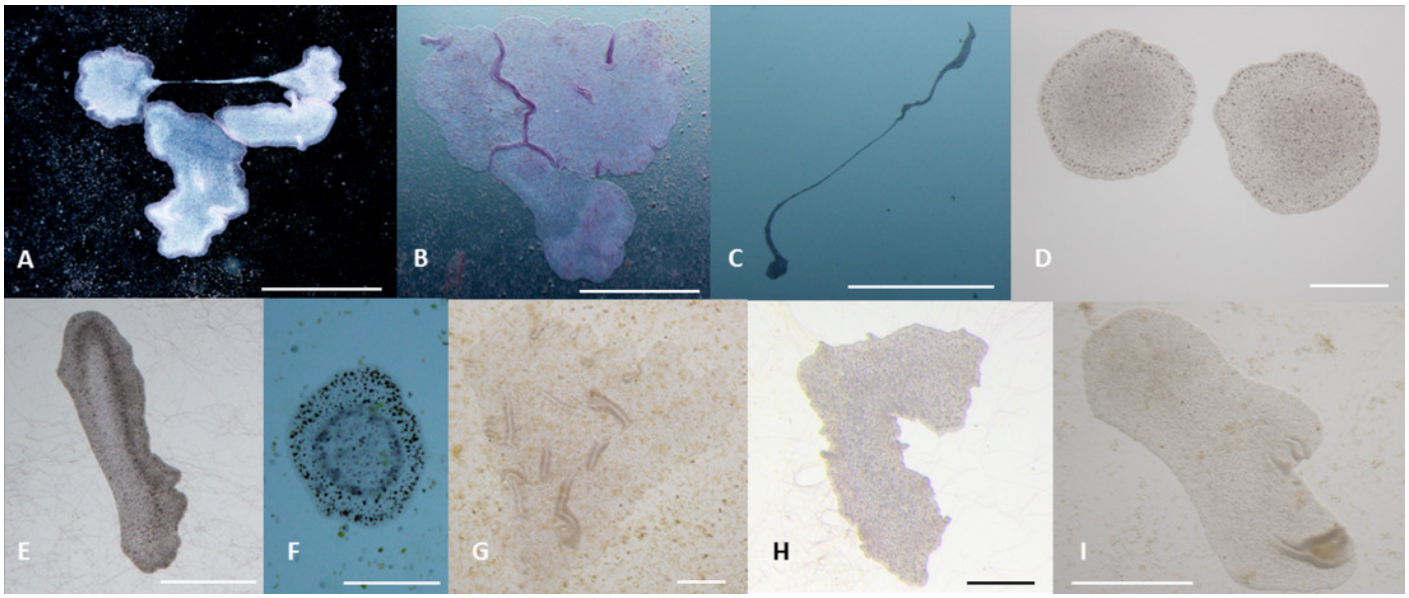

Figure 7 (on next page)

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.

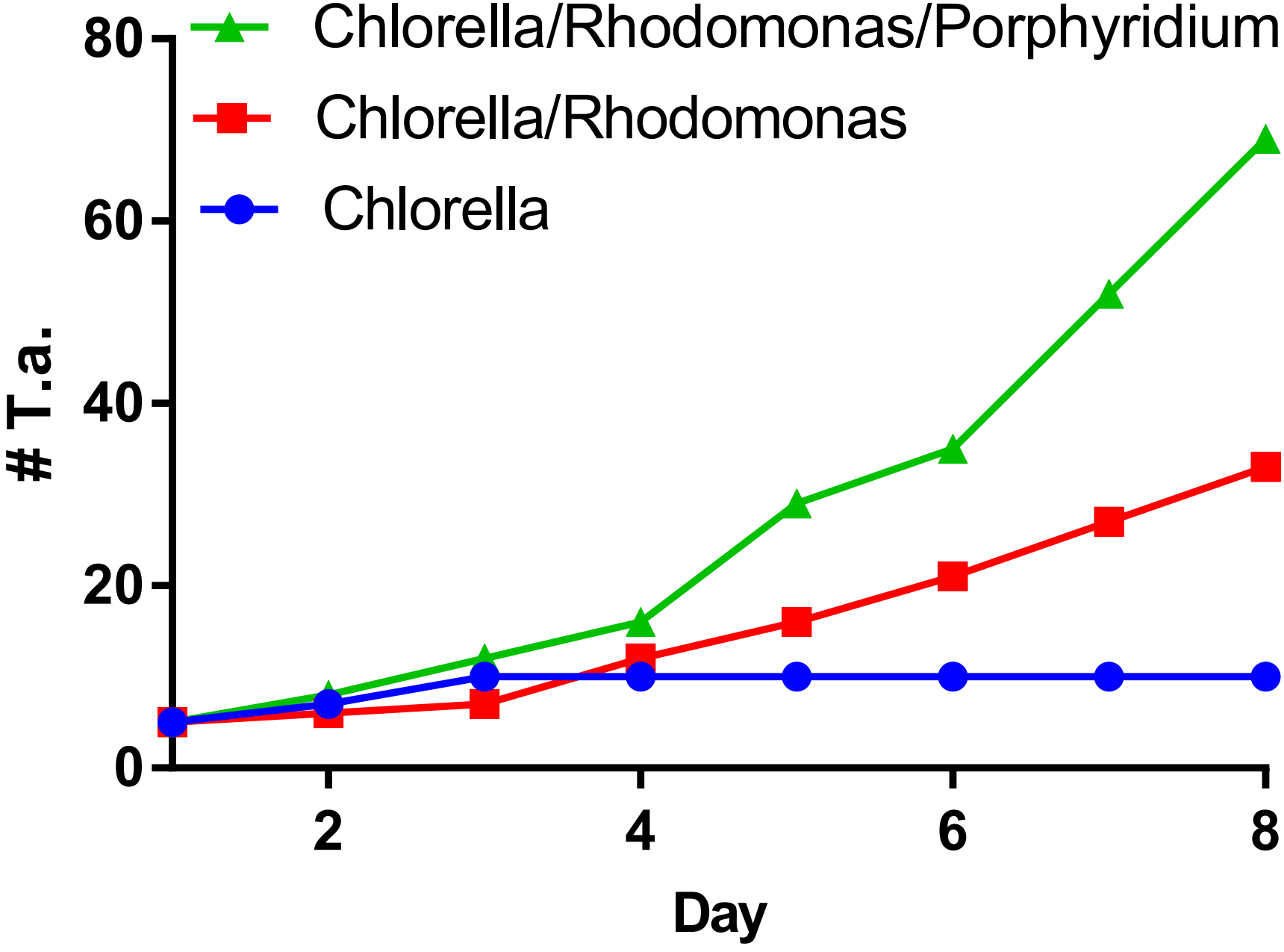

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Figure 8

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 photographed and analyzed using ImageJ program for their number (panel A), mean area (panel B), mean perimeter (panel C), Perimeter/Area ratio (panel D) and 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* 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**.

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