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Rapid Fluorescent Detection of (Anti)androgens with *spiggin-gfp* Medaka

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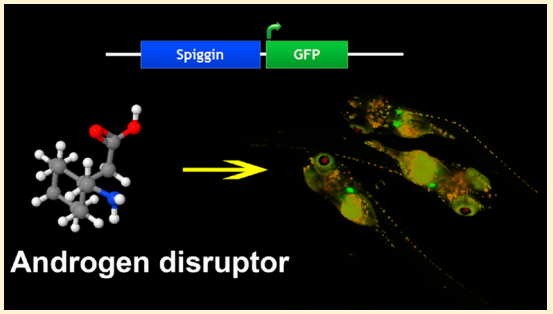
Supporting Information

ABSTRACT: Widespread environmental antiandrogen contamination has been associated with negative impacts on biodiversity and human health. In particular, many pesticides are antiandrogenic, creating a need for robust and sensitive environmental monitoring. Our aim was to develop a sensitive and specific transgenic medaka (*Oryzias latipes*) model bearing an androgen responsive fluorescent reporter construct for whole organism-based environmental screening of pro- and antiandrogens. We analyzed the 5′ regions of the androgen responsive three-spined stickleback (*Gasterosteus aculeatus*) *spiggin* genes in silico, revealing conserved blocks of sequence harboring androgen response elements. Identified putative promoters were cloned upstream of GFP. Germinal transgenesis with *spg1-gfp* led to stable medaka lines. GFP induction was exclusive to the kidney, the site of spiggin protein production in sticklebacks. Significant GFP expression was induced by three or four-day androgen treatment of newly hatched fry, but not by estrogens, mineralocorticoids, glucocorticoids or progestogens. The model responded dose-dependently to androgens, with highest sensitivity to 17MT (1.5 μg/L). In addition to flutamide, the biocides fenitrothion, vinclozolin and linuron significantly inhibited 17MT-induced GFP induction, validating the model for detection of antiandrogens. The *spg1-gfp* medaka model provides a sensitive, specific, and physiologically pertinent biosensor system for analyzing environmental androgen activity.



INTRODUCTION

Antiandrogenic activity in UK river water has been linked to the feminization of wild fish,[1] suggesting a risk to biodiversity. Specifically antiandrogens appear to be acting synergistically with estrogens, resulting in dose-dependent increases in ovo-testes, ovoducts and vitellogenin in male fish. Previous identification of substantial antiandrogenic activity in the River Lambro in Italy suggests that the biological effects observed in the UK are not unique.[2] This is hardly surprising considering that an in vitro study showed that 66/200 pesticides inhibited 5α-dihydrotestosterone (DHT) induced androgen receptor (AR) transcriptional activity.[3] In an unrelated study 37/134 pesticides were found to be antiandrogenic in vitro with some mixtures of pesticides showing a concentration addition relationship,[4,5] resulting in a higher overall antagonistic effect than the most powerful antiandrogen alone. In addition to pesticides, identified sources of androgen axis active chemicals include effluent from wastewater treatment works[6] and kraft pulp mills.[7]

The observed predictability of fish models for mammalian responses to hypothalamic−pituitary−gonadal (HPG) axis active chemicals,[8] suggests that the majority of AR agonists/antagonists active in fish species are also active in humans. Yeast and mammalian cell culture lines transfected with human AR have proved valuable for assessing transactivation potential in humans, but are limited to identifying substances that act directly on the receptor.[9,10] Mouse models for reporter gene based measurement of AR activity have emerged,[11,12] but no aquatic equivalents exist. Despite these tools, definitive proof is lacking that environmental androgen active chemicals are responsible for deleterious effects in humans. However, it is clear that disruption of the androgen axis in humans, particularly within critical developmental periods, can have physiological consequences. For example, modulation of AR

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signaling in humans by synthetic progestins has been linked to breast cancer and exposure of pregnant women to testosterone resulted in reduced sperm count and motility in male offspring.[13,14] Interestingly, all symptoms of testicular dysgenesis syndrome, apart from germ cell cancer, can be reproduced in animal models by exposing them to endocrine disruptors during embryonic or perinatal development.[15] Robust screening models are therefore needed to identify androgen axis disruptors among the thousands of currently untested chemicals in our environment.

In their recent extensive "State of the Science of Endocrine Disrupting Chemicals" document, World Health Organization (WHO) and United Nations Environment Programme (UNEP) highlight improved testing for endocrine disrupting chemicals (EDCs), in particular in high-throughput format, as an important future need.[16] Development of rapid screening tools for AR modulation has been hampered by the lack of specific target genes. Furthermore, the consensus androgen response element (ARE) is identical to that of glucocorticoid, progesterone, and mineralocorticoid response elements,[17] rendering development of specific assays even more arduous.

One of the best-documented physiological responses under tight androgen control is Spiggin production in the three-spined stickleback (*Gasterosteus aculeatus*). In the breeding season, male sticklebacks produce Spiggin glue protein in their kidneys in response to elevated circulating androgen to allow the building of nests. The most effective androgen for inducing Spiggin production is 11-ketostestosterone (11KT).[18] Despite the almost undetectable levels of Spiggin protein in females under natural conditions, females synthesize Spiggin protein in response to exogenous androgens.[19] Androgen-dependent Spiggin synthesis in the stickleback is already used as a biomarker in vivo and in vitro for androgen axis disruption.[20]

The draft directive androgenized female stickleback screen (AFSS) was established by The Organization of Economic Cooperation and Development (OECD) in 2009.[21] It describes the detection of androgens and also, by simultaneously treating female sticklebacks with a reference dose of an androgen (5 μg/L DHT or 0.5 μg/L 17MT), the detection of antiandrogens. The potency of antiandrogens is expressed as equivalents of the model pharmaceutical antiandrogen, flutamide (flutamide equivalents, FL eq). The exposure phase of this test runs for 21 days, followed by dissection of the kidneys of the adult fish to allow quantification of Spiggin protein by enzyme-linked immunosorbent assay (ELISA).[22] During validation of the AFSS flutamide, fenitrothion, vinclozolin, and linuron were used as model antiandrogens.

Here, we describe an alternative, in vivo test based on a fluorescent reporter gene driven by the *spiggin* promoter. Our assay can detect pro- and antiandrogens in medaka larvae 4 days post hatch (dph4), and could serve as a rapid additional screening tool in the testing of chemicals and environmental samples.

MATERIALS AND METHODS

Chemicals and Solutions. Chemicals were purchased from Sigma-Aldrich (MO): 17MT (CAS: 58−18−4), DHT (CAS: 521−18−6), 11KT (CAS: 564−35−2), flutamide (CAS: 13311−84−7), linuron (CAS: 330−55−2), fenitrothion (CAS: 122−14−5), vinclozolin (CAS: 50471−44−8), anastrozole (CAS: 120511−73−1), 17β-estradiol (E_2, CAS: 50−28−2), aldosterone (CAS: 52−39−1), dexamethasone (CAS: 50−02−2), progesterone (CAS: 57−83−0). Medaka medium contained per liter: 10 g NaCl, 0.4 g $CaCl_2.2H_2O$, 0.4 g $MgSO_4.7H_2O$, 0.3 g KCl, 0.02 g methylene blue adjusted to pH 7.2−8.0. Ethyl 3-aminobenzoate methanosulfate salt (MS222, CAS: 886−86−2) was prepared with an equal mass of sodium bicarbonate (CAS: 144−55−8) and adjusted to pH 7.5−8.0.

Characterization and Cloning of *spiggin* Promoters. The in silico analysis of the *spiggin* promoter regions is described in detail in the Supporting Information (SI). Briefly, the *spiggin* gene locus (linkage group IV: 21 000 000- 21 220 000) in the three-spined stickleback genome (BROAD S1 assembly February 2006) was analyzed with Dotter software[23] and PipMaker.[24] mRNA-DNA alignments were carried out using Spidey, and nuclear receptor binding sites were predicted with NHR scan.[25]

Sticklebacks were caught off the Taivassalo shore, Finland. Genomic DNA was extracted using PureLink Genomic DNA mini kit (Invitrogen, CA, USA) and used for PCR of *spiggin* promoters. Primers used in successful promoter amplification, with the added AgeI and XhoI restriction sites underlined, were spg1_for GTCTCGAGCATCTGGTTTTAAGACTGACTGAACCGACGAAG; spg1_rev GTACCGGTTGATGCTGCACGAGAGCAGTTGAAAGAAACAAC; spg4_for ACCTCGAGCAGGTGGGTTCATGCAGTTC; spg4_rev ACACCGGTGAAACAAACAACCCACCACAT.

PCR products were resolved on agarose gel, purified and cloned into TOPO vector (Invitrogen, CA), sequenced prior to excision and ligated into pUC57 with AgeI and XhoI. The eGFP coding sequence from peGFP-1 (Clontech, CA) was excised using AgeI and NotI and ligated into the pUC57-Spg plasmids.

Germline Transgenesis. Briefly, one-cell red-orange strain medaka embryos were injected with a 20 ng/μL nonlinearized solution of plasmid DNA in the absence of meganuclease as previously described by Kinoshita et al. 1996.[26]

EDC Exposure Studies. Exposures were carried out in six-well plates. Stocks were made up in DMSO (final concentration 0.2%). Solutions were renewed every 24 h. Five newly hatched (dph0) F2−F5 *spg1.22-gfp* medaka fry were placed in each well and 12 wells were used for each exposure group giving a total of 60 fry per exposure group. For the estrogen responsive line (Figure 4B): five newly hatched (dph0) F12 *chgh-gfp* medaka fry were placed in each well and 8 wells were used for each exposure group giving a total of 40 fry per exposure group.

Image Capture. Fry were anesthetized with 200 mg/L MS222 and positioned ventrally for imaging of the dorsal region of the abdomen. Fluorescence was imaged using a 120W fluorescence source and ET-GFP long-pass filters (Excitation 480/40, Emission 510LP, Leica Microsystems GmbH, Germany). Images were captured with a 0.3 s exposure time (Figure 3A−C) or 0.75 s (Figure 3D−F and Figure 4) at 8× magnification with an Infinity 1-3C camera (Lumenera Corporation, ON, Canada) fitted to a Leica MZ10F stereomicroscope (Leica Microsystems GmbH, Germany). For the estrogen responsive line (Figure 4B) identical conditions were used except images were captured of the ventral region with a 0.3 s exposure time.

Data Analysis. ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, MD) was used for image analysis. Color images of medaka fry were separated into red, green and blue layers. To remove background fluorescence resulting from endogenous pigments, intensity for each pixel in the red layer was doubled and subtracted from the green layer. This method efficiently removes the endogenous pigmentation appearing red

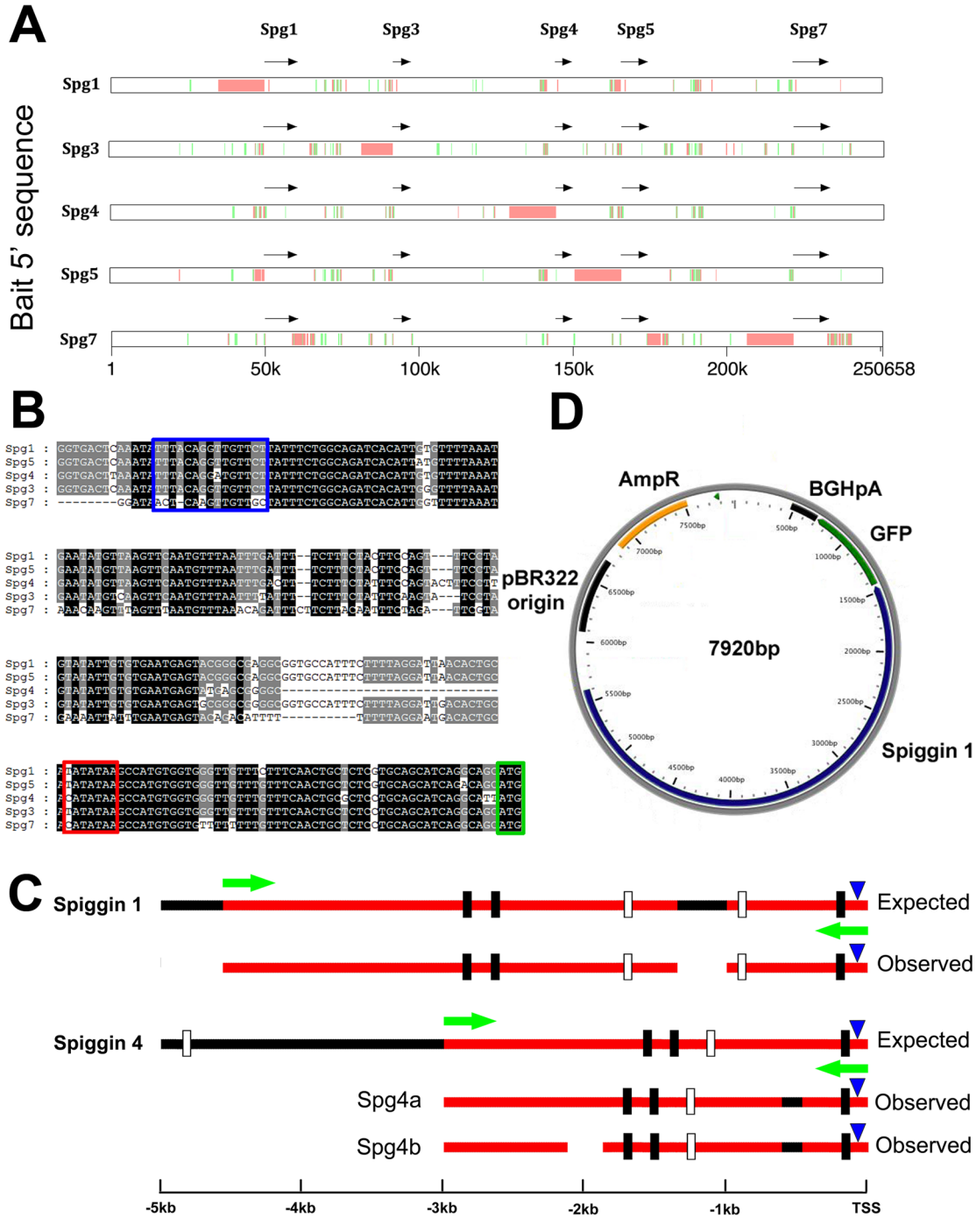

Figure 1. In silico *spiggin* promoter characterization. (A) Overview of the PIPs. Different *spiggin* 5′ regions (baits) were compared to the whole *spiggin* gene locus (20 968 314−21 218 972) (for full PIPs see SI, Figure S3). Arrows denote the location of *spiggin* genes based on mRNA-DNA alignments, and red blocks 100% similarity in sequence to bait. (B) Alignment of *spiggin* 5′ regions upstream of putative ATG (green box). The locations of possible TATA box (red box) and ARE (blue box) are depicted. (C) Schematic presentation of expected (database sequence) and observed (cloned sequence) *spg1* and *spg4* promoters. Predicted ARE (black bars), ERE (white bars), TATA boxes (blue triangles) and primers (green arrows) are shown. Regions predicted as conserved based on the PIPs are shown in red. (D) Plasmid map of the construct used for the *spg1.22-gfp* line.

or yellow in the images captured with GFP long pass filters, leaving the green fluorescence emitted by GFP (SI, Figure S1). The region containing the kidney was manually selected and the mean intensity of these pixels was calculated. In the absence of a means to separate *spg-gfp* transgenic and wild-type fry prior to exposure, all fry were analyzed. Accordingly, only the highest 50% of values were considered from each group.

Statistical Analysis. Statistical analyses were carried out with GraphPad Prism version 5.04 (GraphPad Software, CA). A D'Agostino and Pearson omnibus normality test was carried out on all exposure groups. If data in all groups compared showed normal distribution, then a Student's *t*-test was carried out. Otherwise data were compared using a Mann−Whitney test. Statistical significance is indicated by * $P < 0.05$, ** $P <$

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0.01, *** $P < 0.001$ compared to androgen alone; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to vehicle control; other comparisons are indicated with a bar. Mean ± SEM are given throughout.

RESULTS

Characterization and Cloning of *spiggin* Promoters. The *spiggin* gene in the stickleback genome has been duplicated creating a locus of directed repeats with an estimated copy number of seven (please see SI for more detail).[27] The promoter regions of these genes have not been characterized. We searched for conserved elements within the *spiggin* gene locus with particular focus on the 5′ regions of the genes. DotMatrix plots (SI, Figure S2) and Percent Identity Plots (PIPs) (Figure 1A, SI, Figure S3) suggested that conserved elements are enriched within a region of 5 kb immediately 5′ to the ATG of the *spiggin* genes, and these regions were found to contain conserved ARE and ERE sites (Figure 1A, C, SI, Figure S4). Alignment of the −250 to +3 bp regions to each other revealed a conserved, putative TATA box in addition to candidate ARE in all but *spg7* (Figure 1B). Interestingly, the promoters of *spg1*, *spg3*, and *spg5* all contain an ARE at −203 to −218bp and at −177 to −191bp in the *spg4* promoter (Figure 1B, C) which may suggest that this element is crucial to the AR-mediated activation of the promoters. The position (52−58 bp upstream from the translation start) and sequence (TATA-TAA) of the putative TATA box is conserved in *spg1*, *spg3*, *spg4*, *spg5*, and *spg7*, although in *spg4* and *spg7* the sequence is CATATAA. The number of transcripts on the Ensembl database ranks the *spiggin* genes according to weight of evidence for expression as *spg1* > *spg4* > *spg3* = *spg7* > *spg5* = *spg6*. Based on these data, primers were designed to clone the promoters of *spg1* (4.5 kb), *spg3* (8.0 kb), and *spg4* (2.9 kb) (Figure 1C). A more in-depth description of the promoter analysis results is available as SI.

Promoters of *spg1* and *spg4* were successfully cloned but amplification of *spg3* failed despite various attempts with different primer designs. The cloned promoters showed the highest sequence similarity to expected regions (>98% for *spg1*, >95% for *spg4*). However, the cloned *spg1* promoter had a 399 bp deletion corresponding to a segment that was not predicted as conserved in our analysis (Figure 1C). Sequencing of *spg4* promoter clones revealed two variants, suggesting the presence of two different alleles. Both contained a 140 bp insert compared to database sequence, but *spg4b* displayed an additional 264 bp deletion (Figure 1C). The lengths of the obtained promoters were therefore *spg1* 4160 bp (expected 4513 bp), *spg4a* 3002 bp, and *spg4b* 2747 bp (expected 2869 bp); Genbank accession numbers are KF716169, KF716170, and KF716171, respectively. Despite the observed variation in sequence compared to the database genome sequence, all predicted binding sites were conserved (Figure 1C). All three promoters were cloned upstream of *gfp*. The plasmid map of the construct used in the *spg1-gfp* line is provided in Figure 1D.

Creation of *spiggin-gfp* medaka. Medaka germline transgenesis was carried out, 1322 eggs were injected with *spg1-gfp* and 1021 with *spg4-gfp*, resulting in 73 adult *spg1-gfp* and one adult *spg4-gfp* which had exhibited GFP in the developing embryo. Screening of the *spg4-gfp* adult and 36 *spg1-gfp* adults for germline transmission, by androgen treatment of the F1 progeny, led to identification of seven stable independent lines for *spg1-gfp* and none for *spg4-gfp*. The *spg1-gfp* transgene consisted of 4.159 kb of sequence upstream from the translation start site of the *spiggin1* gene (Figure 1D). Following androgen treatment of the F1 progeny, the *spg1.22-gfp* line was selected as this line demonstrated the strongest GFP signal and the shortest response time (data not shown).

GFP Expression in *spg1.22-gfp* Medaka Adults and Fry. GFP was observed without androgenic treatment in the kidneys of 6-month-old F2 adult male medaka but never in females (Figure 2A, B). Nevertheless, after a seven-day 17MT treatment (302 μg/L) of F4 adult females, GFP expression was observed in the kidneys (SI, Figure S5).

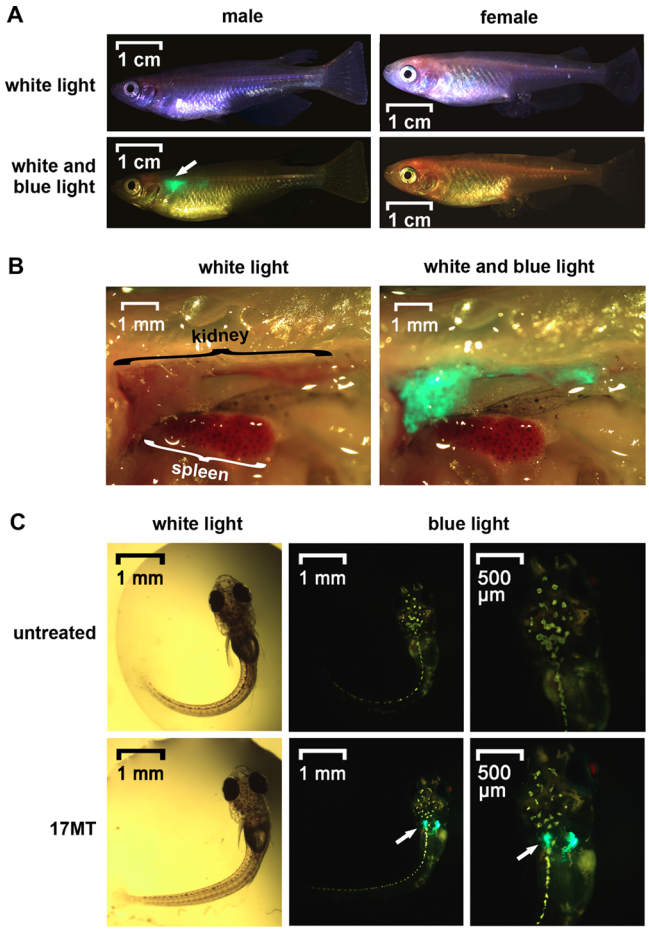

Figure 2. *Spg1.22-gfp* transgenic medaka. (A) Untreated, adult *spg1.22-gfp* fish under white light with or without additional blue light from a fluorescence source. Arrow denotes *gfp* expression in the kidneys. (B) Dissected untreated adult male medaka observed under white light with or without additional blue light from a fluorescence source. (C) Dph4 *spg1.22-gfp* fry treated for 4 days with vehicle or 17MT (302 μg/L) visualized under white and blue light. Arrows point to *gfp* expression in the developing mesonephros.

No GFP was visible in untreated F1 fry of the *spg1.22-gfp* line until dph60, around the time when medaka become reproductively active, suggesting that the expression of *gfp* is induced by increasing levels of androgens. Consequently, we screened F1 fry with a high concentration of androgen (17MT, 302 μg/L) to confirm germ line transmission. This treatment resulted in *gfp* expression in the developing mesonephros (Figure 2C). All further experiments were carried out using unsorted F2−F5 *spg1.22-gfp* fry resulting from a nontransgenic (red-orange strain)/ transgenic cross, exposed from dph0

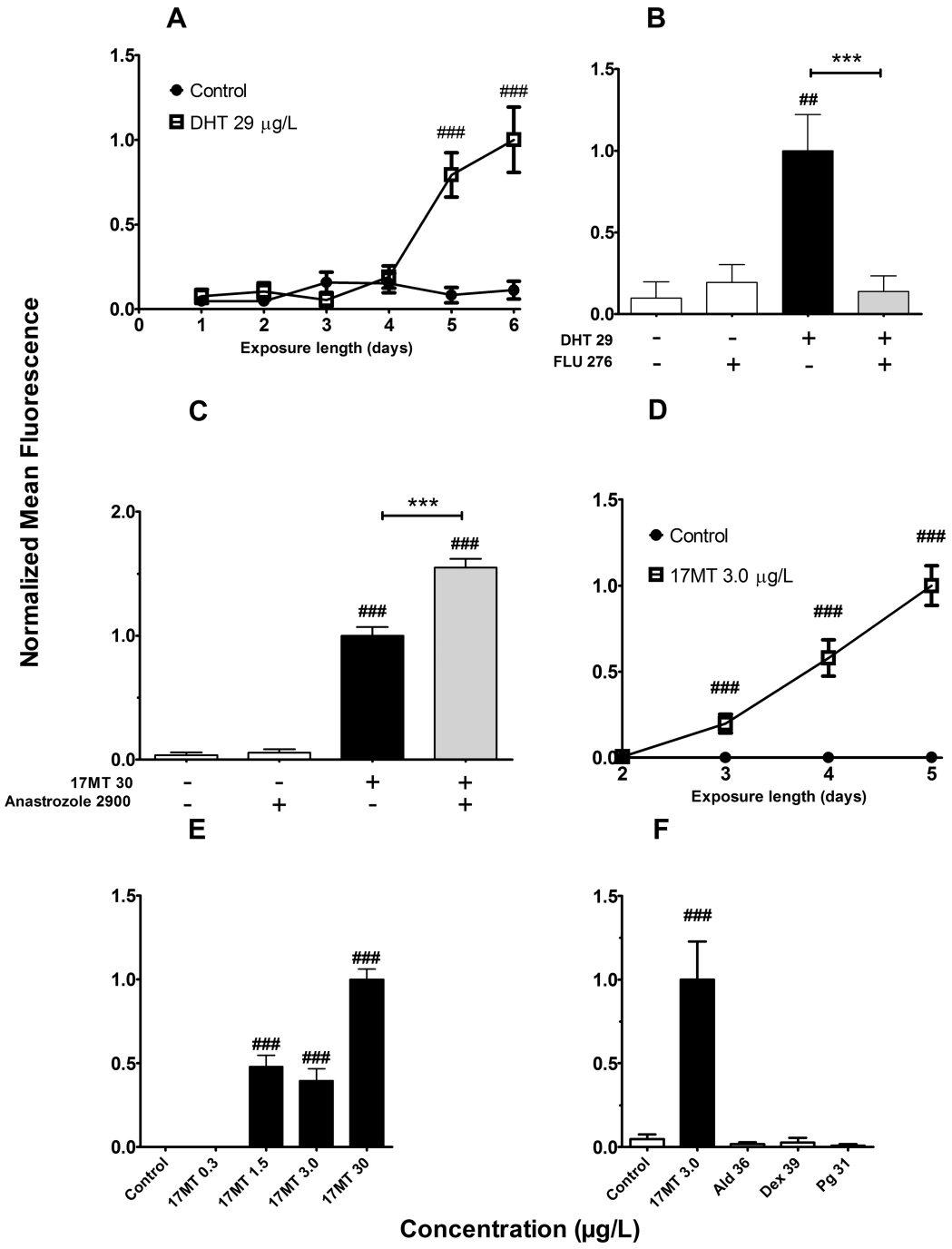

Figure 3. Androgenic specificity of the *spg1.22-gfp* line. (A) Evolution of fluorescent signal during exposure to DHT. (B) Inhibition of DHT induced fluorescent signal after 6 days of cotreatment with flutamide. (C) The effect of anastrozole on GFP signal induced following 3 days exposure to 17MT. (D) Evolution of fluorescent signal during exposure to 17MT 3.0 μg/L. (E) Dose-response relationship after 4 days of exposure to 17MT. (F) Quantification of GFP signal following a 4 day treatment with aldosterone (Ald), dexamethasone (Dex) or Progesterone (Pg).

onward. All groups contained approximately 50% transgenic and 50% wild-type fry.

GFP Induction with Androgens. GFP was significantly induced in fry exposed to 29 μg/L of the nonaromatizable androgen DHT from hatching to dph5, showing that *spg1-gfp* can be induced by androgens (Figure 3A).

To determine specificity of the fluorescent response, fry (dph0) were cotreated for 6 days with DHT (29 μg/L) and/or the pharmacological antiandrogen flutamide (276 μg/L). DHT induced GFP production was entirely abolished by cotreatment with flutamide, showing that the DHT effect is mediated via AR (Figure 3B).

A 72 h exposure to the pharmacological aromatase inhibitor anastrozole (2.9 mg/L) did not induce fluorescence (Figure 3C). However, the aromatizable androgen 17MT (30 μg/L) did induce significant fluorescence and cotreatment with 17MT and anastrozole induced a 1.5-fold higher signal than 17MT alone. This suggests that blocking the conversion of 17MT to methylestradiol by inhibition of aromatase maintains a greater degree of androgen signaling.

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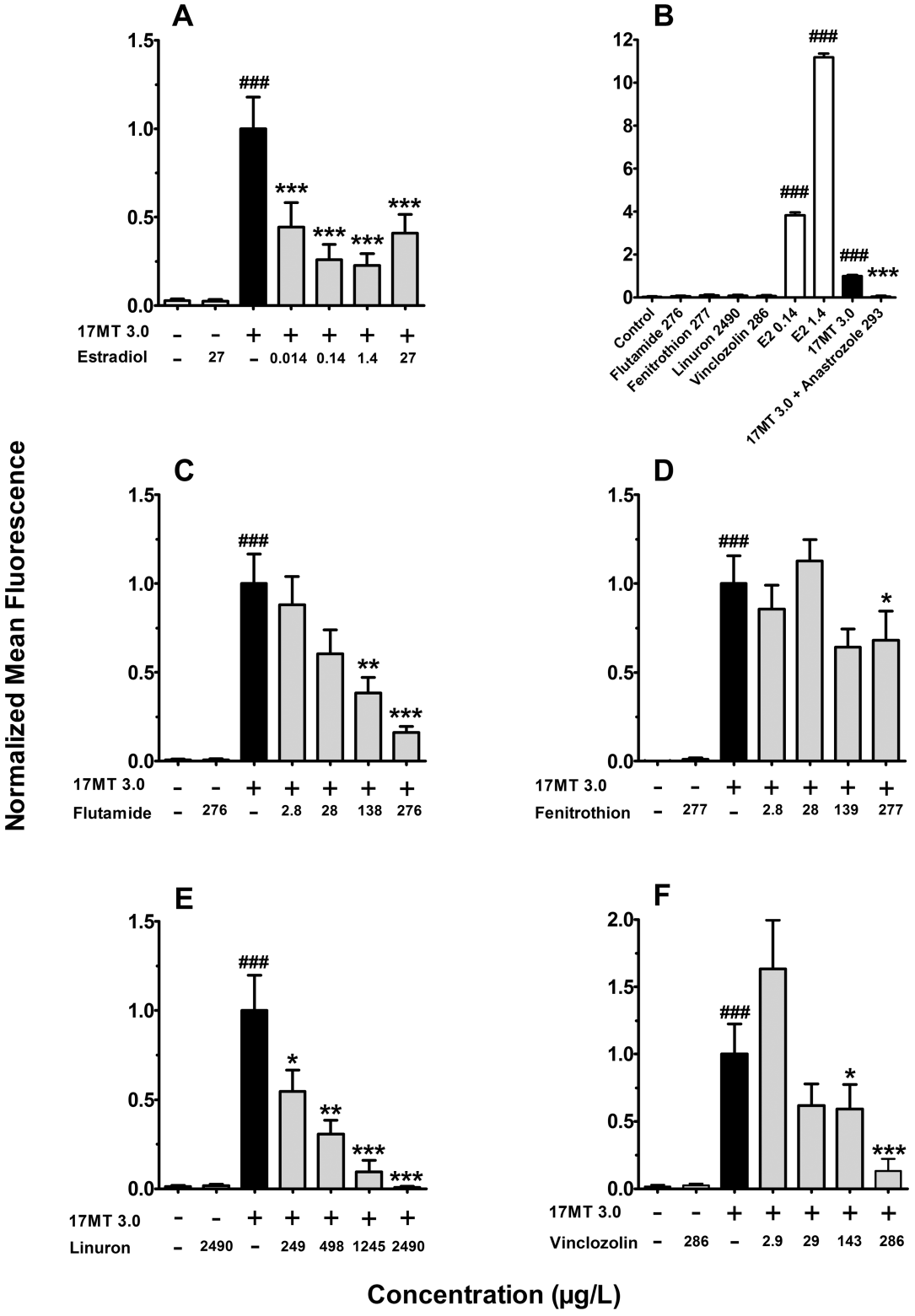

Figure 4. Antiandrogen detection assays with 17MT. (A) Quantification of GFP signal induced by E_2 alone, or in cotreatment with 17MT. (B) Lack of estrogen axis activation following 4 day treatment of *chgh-gfp* medaka with high concentrations of flutamide, fenitrothion, linuron and vinclozolin. (C–F) The effects of a 4 day cotreatment of *spg1.22-gfp* fry with 17MT and a range of concentrations of flutamide, fenitrothion, linuron, or vinclozolin.

Optimized Protocol. Due to the more rapid response observed with 17MT compared to DHT (compare Figure 3A and C), 17MT was selected as the cotreatment for the detection of antiandrogens. We conducted a time course experiment with 3.0 µg/L 17MT from dph0–5 (Figure 3D). A statistically significant increase in GFP signal was induced after 72 h exposure, however, a more robust GFP signal was observed after 96 h, therefore, further exposures were carried out for 96 h.

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Using this protocol, 17MT was tested at four concentrations (Figure 3E). The lowest observable effect concentration (LOEC) for 17MT was 1.5 μg/L.

Specificity of the Response. In addition to androgens, mineralocorticoids, glucocorticoids, and progestogens have been shown to induce some AREs. Therefore, the specificity of the response for AR agonists was tested using aldosterone, dexamethasone, and progesterone, respectively. A lack of GFP production following treatment with 10^{-7} M of each substance from dph0 to dph4 demonstrated the specificity of the response. Significant fluorescence was induced with a 10-fold lower concentration of 17MT (Figure 3F).

Estrogens have been reported to reduce androgen-stimulated spiggin response in adult stickleback fish in vivo and in stickleback kidney cells in vitro.[20,28] The *spg1* promoter also contains putative EREs. We therefore tested the effect of E_2 on 17MT-induced GFP production with our medaka model. GFP signal was observable in fry exposed to 17MT (3 μg/L) for 4 days (Figure 4A). Co-treatment with E_2 (14 ng/L-27 μg/L) repressed androgen-induced *gfp* expression, whereas E_2 alone had no observable effect.

Detection of AR Antagonists Using *spg1.22-gfp*. Due to the observed repression of 17MT-induced *gfp* expression by E_2, prior to determining the ability of the *spg1.22-gfp* line to identify antiandrogenic activity, we verified that the test substances were not estrogenic. Using an estrogen responsive transgenic medaka line harboring the promoter of the *choriogenin H* gene driving expression of *gfp* (*chgh-gfp* line), we carried out exposures within the same developmental window (96 h, dph0-dph4) and under the same exposure conditions as for the *spg1.22-gfp* line. This line was created using the same genetic construction described by Karauchi et al. for the creation of their line.[29,30] All four antiandrogens (flutamide, fenitrothion, linuron, and vinclozolin) failed to induce *gfp* expression compared to the solvent control group, when tested at the maximum concentration to be utilized in the proceeding experiments (Figure 4B). In contrast E_2 strongly induced a fluorescent response as did 17MT to a lesser extent. The 17MT-induced fluorescent response was completely abolished when cotreated with anastrozole.

To determine the sensitivity of a four-day assay for the detection of antiandrogens, we first tested flutamide. A concentration dependent inhibition of GFP signal was observed at all concentrations tested. Flutamide significantly inhibited GFP signal at ≥138 μg/L (Figure 4C).

We then tested the assay sensitivity for three well-characterized environmentally relevant antiandrogens. Fenitrothion significantly reduced GFP signal at 277 μg/L and almost at 139 μg/L ($P = 0.0657$ cf. 17MT alone) (Figure 4D). Linuron and vinclozolin significantly reduced the GFP signal at ≥249 μg/L (Figure 4E) and ≥143 μg/L (Figure 4F) respectively.

Therefore, the determined LOECs were 138 μg/L (flutamide), 277 μg/L (fenitrothion), 249 μg/L (linuron) and 143 μg/L (vinclozolin). This indicates the following order of antiandrogenic potency in terms of molarity flutamide = vinclozolin > fenitrothion = linuron.

DISCUSSION

Here, we describe an in vivo test for the detection of androgenic/antiandrogenic chemicals. Our aim was to create a medaka transgenic line suitable for detection of pro- and antiandrogenic compounds among industrial chemicals or present in environmental samples. The *spiggin* response from the three-spined stickleback, which is under validation as an OECD guideline for testing of androgen axis disruptors (OECD 2009a), was modeled here in medaka using the *spiggin1* promoter driving expression of *gfp*. We believe that this tool will be useful for screening and prioritizing purposes, before carrying out more expensive and labor-intensive guideline tests.

The three-spined stickleback *spiggin* gene response is well-tested and androgen-specific.[21,31] Interestingly, we identified a putative ARE with a conserved sequence (TTTA-CAggtTGTTCT) and position (−203 to −218bp) in *spg1*, *3*, and *5*. This element resembles the classical inverted ARE (AGAACAnnnTGTTCT) but displays changes in the first half site, of which the G → T change in position −6 in particular may have consequences for functionality.[32] It remains to be studied whether this element mediates the androgen-specific induction of the *spiggin* promoter. It is also important to note that the conserved regions between *spiggin* gene promoters extend up to −5kb from the ATG, suggesting the presence of other sequences of regulatory importance.

We confirmed that the transgene drives kidney-specific expression of *gfp* mimicking the physiological expression of *spiggin* observed in stickleback, possibly due to a kidney-specific transcription factor binding site being present within the promoter. This transcription factor must be sufficiently conserved between species to confer the same tissue specificity. In addition, the male specific expression of *spiggin*[18] is faithfully mimicked in our transgenic medaka model. We show that in response to AR agonists, GFP is produced in the kidneys and is quantifiable in fry immediately after hatch. This method is noninvasive as fluorescence quantification can be carried out in intact, live fry. Interestingly, *spg1.22-gfp* fry appear to be less sensitive to aquatic exposures with DHT than 17MT. This is in accordance with the AFSS assay[21] which is conducted with androgen cotreatments of either 5 μg/L DHT or 0.5 μg/L 17MT. The need for a higher concentration of DHT is hypothesized to be due to low bioavailability or stability.

With this in mind, we developed a four day protocol for the detection of antiandrogens by cotreating with 17MT. We determined that the sensitivity of our assay is 138 μg FL eq/L, less than half the LOEC that induces testis-ova in medaka[33] and reduces secondary sexual characteristics in stickleback.[34] This is sufficiently sensitive to detect environmentally relevant antiandrogenic activity, given levels found in river samples, for example, Lambro River, Italy (370−4723 μg FL eq/L)[2] as well as effluent from wastewater treatment plants (21.3−1231 μg FL eq/L in the UK)[6] and even some control sites (>150 μg FL eq/L in the River Ock, UK).[35]

We achieved comparable flutamide sensitivity to the AFSS (138 μg/L and 10−250 μg/L respectively).[21] We expect a higher sensitivity to be achieved with a homozygous line, which have previously decreased interindividual differences in response caused by different copy numbers and genetic locations of the transgene in other transgenic models (data not shown). The "near miss" with 28 μg/L flutamide ($P = 0.0588$ cf. 17MT alone) suggests that, with a tighter interindividual response, a greater sensitivity to flutamide can be achieved. Interestingly the LOECs determined here for environmental antiandrogens were also similar to those obtained during interlaboratory testing of the AFSS (fenitrothion: 277 μg/L cf. 60 μg/L, linuron: 249 μg/L cf. 250 μg/L and vinclozolin: 143 μg/L cf. 100 μg/L, respectively). We observed the following order of potency: flutamide =

vinclozolin > fenitrothion = linuron, which compares favorably with results obtained for the AFSS interlaboratory testing (fenitrothion > flutamide = vinclozolin > linuron)[21] with the exception of fenitrothion. Interestingly, E_2 inhibited 17MT-induced activation of the *spg1* promoter in a concentration-dependent manner. This fits with observations of inhibition of spiggin production in vitro in stickleback kidney cells with 270 μg/L E_2[20] and in vivo in male stickleback with 20 ng/L ethinylestradiol,[28] although the in vivo study failed to reach statistical significance. The mechanism of the E_2 mediated inhibition of Spiggin production remains unclear. Negative feedback through the HPG axis is unlikely to be the only mechanism as the effect has been observed in vitro, although at much higher concentrations.[20] As proposed previously[20,28] the effect may be local, acting e.g. at the level of AR expression regulation, through membrane bound ER or through the putative EREs in the spiggin promoters. Due to the well-described induction of aromatase expression by estrogens[36] it is entirely possible that all estrogens are also antiandrogenic in vivo, inducing aromatase expression, which in turn converts androgens to estrogens—reducing androgen axis signaling. In light of this finding, it is possible that the increase in GFP production observed with 17MT plus anastrozole compared to 17MT alone (Figure 3C) is due to a combinatorial effect. This may be due to an increase in available androgen leading to an increase in GFP production and/or a decrease in inhibition of *spiggin* transactivation by methylestradiol, if indeed it inhibits GFP production as we have observed with E_2.

As a screening tool prior to higher tier tests, our assay will reduce exposure length from 21 to 4 days, translating into a vast reduction in cost and turnaround time, making it ideal for adaptation to medium throughput screening. The ability to perform this assay prior to protected life stages considered as laboratory animals in Europe is an additional ethical advantage.[37] Finally, the use of the *spg1.22-gfp* medaka line is expected to facilitate interlaboratory comparisons as the AFSS assay suffers from variable sensitivity due to differences in the ages and maturities of the adult stickleback used.[21]

Medaka offer certain advantages over other fish species such as their adaptability to salt or fresh water allowing this model to be used to test unextracted, whole water sample from the entire length of a river, from its source to its estuary and out to sea. This will allow comparisons of androgen axis activity along rivers in an in vivo model and may lead to the identification of point sources of pollution. Additionally, medaka possess a well-characterized XX/XY sex determination system with the master sex determining gene, *dmy*, present on the Y chromosome[38,39] allowing disparities between genetic and phenotypic sex to be linked to levels of androgen axis activity.

The experiments presented here were all carried out with transgenic medaka fry resulting from a cross between wild-type and heterozygous parents. This resulted in the production of an approximately equal mix of wild-type and heterozygous fry. As no basal fluorescence is observable in the fry of this line, it was impossible to exclude wild-type fry prior to exposure and all statistical analyses were carried out on the mixed population. A major goal in the near future is, therefore, the creation of a homozygous line. This feature was not achieved prior to this series of experiments as the procedure can take several years, but is ongoing. The transmission rate observed in all generations of *spg1.22-gfp* medaka used for this study was approximately 50%, suggesting a single transgene insertion site. However, it would require two additional generations before obtaining homozygous fish, then a number of generations to reamplify the population to a level allowing sufficient egg production for large-scale experiments.

It can realistically be envisaged that availability of a simple, rapid and relatively inexpensive in vivo test for androgen axis activity will lead to an increase in the number of raw chemicals and environmental samples tested. This should result in an increased identification of pro- and antiandrogenic EDCs and provide opportunities for regulatory authorities to reduce or eliminate human and wild-life exposure to these substances.

Spg1.22-gfp medaka fry provide a unique model that allows rapid detection and quantification of androgenic and antiandrogenic substances in the environment. Compared to the current OECD AFSS guideline, our model reduces the detection time from over 21 days to 4 days while maintaining sensitivity and eliminating labor-intensive steps (dissection, sample preparation, and ELISA). We consider that this model is adapted to environmental monitoring as well as medium-throughput screening of chemicals prior to higher tier tests such as the AFSS.

■ ASSOCIATED CONTENT

Supporting Information

A more in-depth description of the promoter analysis results is available as supporting text, as well as five figures as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Two of the authors (G.L. and B.D.) have financial interests in WatchFrog, which is planning to commercialize the test described here. A.S., P.S., A.T., D.D.P. and G.L. are currently employed by WatchFrog, N.M and P.D. were employees of WatchFrog when they were involved in this study. The other authors declare no competing interests.

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