Environmental Science & Technology

ARTICLE

pubs.acs.org/est

Effects of Triclocarban, Triclosan, and Methyl Triclosan on Thyroid Hormone Action and Stress in Frog and Mammalian Culture Systems

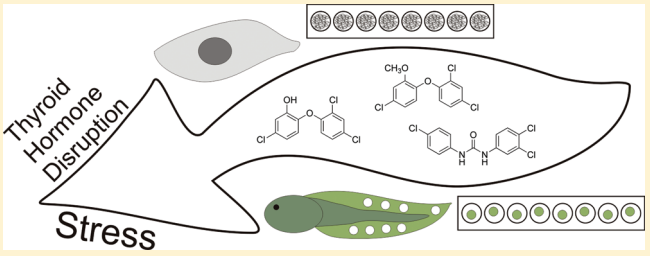
Ashley Hinther,[†] Caleb M. Bromba,[‡] Jeremy E. Wulff,[‡] and Caren C. Helbing*[,†]

[†]Department of Biochemistry & Microbiology, University of Victoria, P.O. Box 3055 Stn CSC, Victoria, B.C., Canada, V8W 3P6

[‡]Department of Chemistry, University of Victoria, P.O. Box 3065 Stn CSC, Victoria, B.C., Canada, V8W 3 V6

Supporting Information

ABSTRACT: Triclosan (TCS) and triclocarban (TCC) are widely used broad spectrum bactericides that are common pollutants of waterways and soils. Methyl triclosan (mTCS) is the predominant bacterial TCS metabolite. Previous studies have shown that TCS disrupts thyroid hormone (TH) action; however, the effects of mTCS or TCC are not known. The present study uses the cultured frog tadpole tail fin biopsy (C-fin) assay and the TH-responsive rat pituitary GH3 cell line to assess the effects of these three chemicals (1−1000 nM) on TH signaling and cellular stress within 48 h. mRNA abundance of TH receptor β, *Rana* larval keratin type I (TH-response), heat shock protein 30, and catalase (stress-response) was measured using quantitative real-time polymerase chain reaction in the C-fin assay. The TH-responsive gene transcripts encoding growth hormone, deiodinase I, and prolactin were measured in GH3 cells with the heat shock protein 70 transcript acting as a cellular stress indicator. We found alteration of stress indicators at a wide range of concentrations of TCS, mTCS, and TCC in both test systems. mTCS and TCC affected TH-responsive gene transcripts at the highest concentration in mammalian cells, whereas a modest effect included lower concentrations in the C-fin assay. In contrast, TCS did not affect TH-responsive transcripts. These results identify nontarget biological effects of these bacteriocides on amphibian and mammalian cells and suggest the TH-disrupting effects observed for TCS could be mediated through its metabolite.



INTRODUCTION

Triclosan (TCS) and triclocarban (TCC) (Figure S1 of the Supporting Information) are broad-spectrum, synthetic, lipophilic, antimicrobial agents used in a variety of personal care products (PCPs). TCS was originally introduced in 1972 as an active ingredient in a surgical scrub for professional health care and is now used in antibacterial mouthwash and toothpaste, detergents, shampoos, deodorants, plastic cutting boards, sports equipment, textiles, and furniture, whereas TCC is mostly added to antibacterial soaps and body washes.[1−3] TCS works through the inhibition of the enzyme enoyl-acyl carrier-protein reductase, which catalyzes an essential lipid biosynthesis step in membranes of Gram-positive and Gram-negative bacteria, as well as in many types of fungi.[4] TCC acts predominantly against Gram-positive bacteria.[4−6]

TCC typically accounts for 2% by weight of antimicrobial soaps, whereas TCS accounts for a lower amount in the range of 0.1−0.3% by weight although some formulations used in hospitals can be as high as 5%.[2] Approximately 45% of liquid and bar soaps contain these antimicrobials and it is estimated that approximately 1 million pounds of TCC are produced for the U.S. market per year.[7,8] Because of the large amount of use of these antimicrobials, there is widespread environmental contamination. TCS and TCC have been detected in wastewater effluent in quantities up to micromolar (μg/L) concentrations in North America, Europe, and Asia.[9−12] Not only are humans exposed to these antimicrobials through environmental contamination but also TCS and TCC are also readily absorbed through the gastrointestinal tract, skin, and oral mucosa.[13−15] It has been reported that 0.39% (0.54 mg) of an average 138 mg of TCC applied to the entire body was absorbed after a typical whole-body shower lather.[14] TCS has been found at levels as high as 2000 μg/kg lipid in human breast milk and has also been found in urine samples from young girls.[16−18]

Not only can TCS bioaccumulate in animal tissues but also its biological metabolite, methyl TCS (mTCS, Figure S1 of the Supporting Information), has been detected in fish exposed to wastewater.[19] Methyl TCS is an environmental methylation product of TCS; however, the environmental fate of TCS and mTCS are quite different, with TCS being easily degraded by photolysis and mTCS being much more persistent.[11]

TCS is structurally similar to thyroid hormone (TH) (Figure S1 of the Supporting Information) and there is evidence that it affects thyroid-mediated processes. Exposure of *Rana catesbeiana* tadpoles to TCS resulted in accelerated TH-dependent

Received: December 14, 2010
Accepted: May 3, 2011
Revised: May 2, 2011
Published: May 16, 2011

metamorphosis as well as altered the levels of TH-responsive mRNA transcripts in tail and brain tissue.[20] *Xenopus laevis* XTC-2 cells coexposed to TCS and TH showed an increase in the mRNA levels of the TH receptors, TRα and *TRβ*.[20] Moreover, *Xenopus laevis* premetamorphic tadpoles exposed to TCS through prometamorphosis exhibited an increase in *TRβ* transcript levels in stage-matched tadpoles at metamorphic climax.[21] This same study also found tadpole development was significantly accelerated upon exposure to TCS although thyroid morphology and thyroxine levels were unchanged (ref 22 for further information). In mammals, TCS decreased the levels of thyroxine (T_4) and triiodothyronine (T_3) but did not affect the levels of thyroid stimulating hormone (TSH) when fed to rats.[23−25] Evidence suggests TCS may upregulate hepatic catabolism of THs, through activation of the nuclear receptor, pregnane X receptor (PXR), which may lead to decreased levels of circulating T_4 and T_3.[26] No studies have been done examining the effect of mTCS on TH signaling in amphibia or mammals.

In addition to TR and PXR, recent studies also found evidence TCC and TCS disrupt steroid hormone action. TCC enhanced testosterone-induced transcription and increased the size of male sex accessory organs in castrated male rats when they were fed both testosterone and TCC.[27] TCC also potentiated the estrogen receptor α (ERα)-mediated transcriptional activity induced by estradiol.[27] TCS was shown to be a weak androgen when tested in *Oryzias laptipes*.[28] Because TCC altered the transcriptional activity of the androgen and ERα receptor, it is plausible that signal transduction systems modulated by other members of the steroid/TH nuclear receptor superfamily could also be affected.[27]

Our laboratory recently developed a screening assay, which allows rapid screening of potential endocrine disrupting chemicals. The in vitro cultured tail fin biopsy (C-fin) assay uses cultured premetamorphic *Rana catesbeiana* tadpole tail fin biopsies that are responsive to TH exposure.[29,30] In the present study, we used quantitative real-time polymerase chain reaction (QPCR) to measure the effects of TCS, mTCS, and TCC on frog tail fin biopsies and rat pituitary GH3 cells. Disruption of TH action in the C-fin assay was assessed by measurement of the mRNA abundance of TH-induced TH receptor *β* (*TRβ*) and TH-repressed *Rana* larval keratin type I (*RLKI*).[31] Not only did we want to investigate the endocrine disrupting effects these chemicals may have but also we also wanted to determine if these chemicals were also eliciting a cellular stress response, which could impact TH signaling or indicate altered cellular function. We therefore evaluated the transcript levels of the heat shock protein 30 (*HSP30*) and catalase (*CAT*). Indicators of TH action in GH3 cells were mRNAs from known TH-regulated genes: growth hormone (*Gh*),[32] deiodinase I (*Dio1*),[33] and prolactin (*Prl*),[34] and heat shock protein 70 (*Hsp70*) transcripts served as a cellular stress marker.[35]

MATERIALS AND METHODS

Experimental Animals. Premetamorphic *Rana catesbeiana* tadpoles were caught locally (Victoria, BC) or purchased from Ward's Natural Science Ltd. (St. Catherines, ON). Taylor and Kollros (TK) [36] stage VI−VIII animals were used. Animals were housed in the University of Victoria aquatics facility and maintained in 100 gallon fiberglass tanks containing recirculating water at 12 °C with exposure to natural daylight. Tadpoles were fed daily with spirulina (Aquatic ELO-Systems, Inc., FL). Animals used in this study were treated and maintained in accordance with the guidelines of the Canadian Council on Animal Care.

Test Chemicals. Triclosan (5-chloro-2-[2,4-dichloro-phenoxy]-phenol; TCS; CAS 3380−34−5; ≥97% pure; Sigma-Aldrich Canada Ltd., Oakville, ON) was prepared in 400 μM NaOH and Triclocarban (3,4,4′-trichlorocarbanilide or 3-(4-chlorophenyl)-1-(3,4-dichlorophenyl)-urea; TCC; CAS 101−20−10; 99% pure; Sigma-Aldrich) was prepared in dimethyl sulfoxide (DMSO). Methyl TCS (mTCS) was synthesized as described in the Supporting Information.

To better equalize the amount of active ingredient in each of the chemicals tested, we applied equimolar concentrations of each of the chemicals rather than by weight. Therefore, the application range equivalents for TCS and mTCS applied at 1−1000 nM were 290 ng/L to 290 μg/L and 304 ng/L to 304 μg/L respectively and for TCC applied at 10−1000 nM were 0.316 μg/L to 316 μg/L. The TCS concentrations used were equivalent to those used previously in whole animal exposures.[20] All test chemical stocks were prepared as 100× concentrates through serial dilutions in the indicated solvents (solvent concentration was kept constant) and stored at −20 °C.

Organ and Cell Culture. Preparation of the tail fin biopsy cultures was adapted from conditions described previously.[29,30] A rat GH3 cell line originating from the pituitary was purchased from American Type Culture Collection (ATCC; Manassas, VA). Further details can be found in the Supporting Information.

Isolation of RNA and Quantification of Gene Expression. RNA was isolated and transcript levels analyzed using QPCR as described in Table S1 of the Supporting Information. All data sets were analyzed with nonparametric statistics as described in the Supporting Information.

RESULTS

Amphibian Organ Culture Exposures. In a previous study, we showed that in vivo exposure of premetamorphic *Rana catesbeiana* tadpoles to TCS accelerated TH-dependent postembryonic development and enhanced TH-dependent induction of gene expression in a cell line derived from *Xenopus laevis*.[20] We chose concentrations of TCS and, by extension, mTCS comparable to these earlier studies. The TCC concentrations were chosen as a compromise between observations with TCS above and those from a previous study on mammalian cells.[27] In the C-fin assay, four of the eight tail biopsies from each individual tadpole were exposed to solvent control and three concentrations of TCS (1, 10, and 100 nM), mTCS (1, 10, and 100 nM), or TCC (10, 100, and 1000 nM). The median values for the *rpL8* normalizer transcript were highly comparable between treatments suggesting that RNA integrity was not adversely compromised by treatments (Table S2 of the Supporting Information). *TRβ* and *RLKI* transcript levels were not significantly affected by TCS treatment alone (part A of Figure 1). The *TRβ* transcript was significantly increased at 10 nM mTCS by 1.4-fold (p = 0.028, Wilcoxon) relative to vehicle control, whereas *RLKI* transcripts were marginally increased upon exposure to 1 nM mTCS by 1.3-fold (p = 0.050, Wilcoxon) relative to vehicle control (part A of Figure 1). TCC exposure did not affect *TRβ* transcript levels, whereas *RLKI* transcript levels were significantly decreased (p = 0.021, Kendall's W on the control plus TCC only

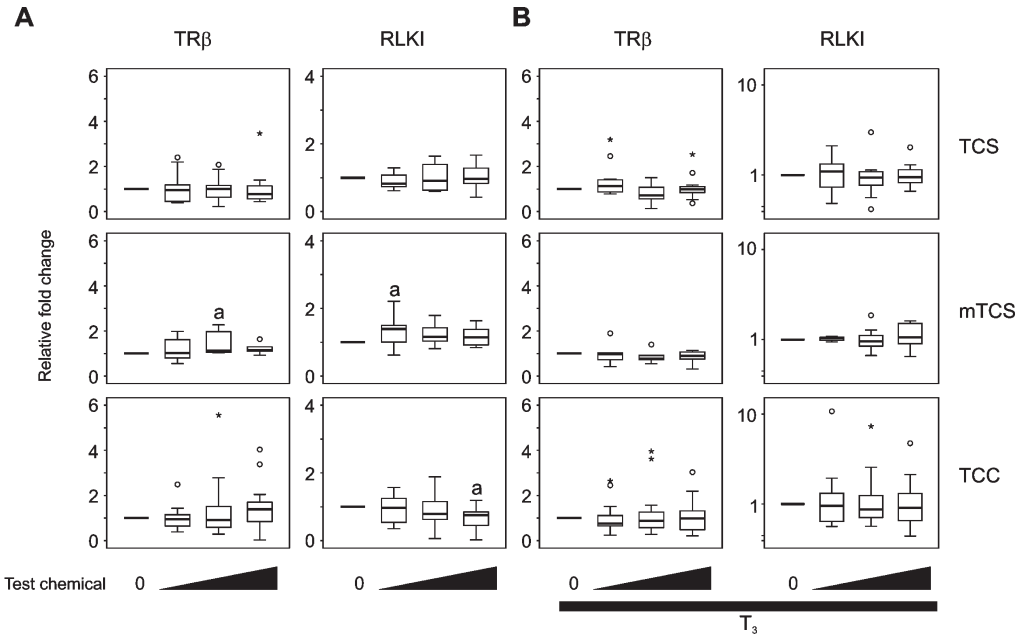


Figure 1. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin I (*RLKI*) transcript levels in the C-fin assay after exposure to TCC, mTCS, and TCC in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n = 8−24) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were as follows: 1, 10, and 100 nM TCS or mTCS, and 10, 100, and 1000 nM TCC. The results are expressed as fold change relative to the vehicle control (A) or to the T_3-induced levels (B) as described in the Materials and Methods section. The medians are shown as solid black lines within the box, and the box indicates the first and third quartiles. The whiskers indicate minimum and maximum values. Outlier (cases between 1.5 and 3.0 box lengths from the upper or lower edge of the box) and extreme values (cases >3.0 box lengths from the upper or lower edge of the box) are indicated by an open circle and asterisk, respectively. Increasing concentrations of test chemicals are represented by bevels. Significance is indicated by an 'a' ($p \leq 0.05$ relative to control in "A" or relative to the T_3-induced response in "B", Wilcoxon).

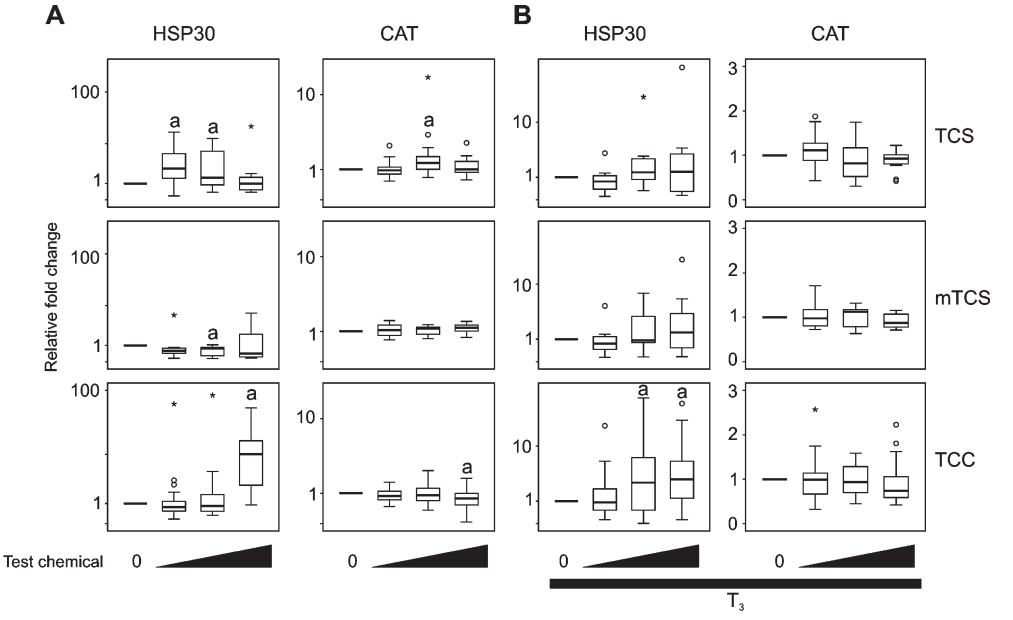


Figure 2. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to TCS, mTCS, and TCC in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n = 8−24) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were as follows: 1, 10, and 100 nM TCS or mTCS, and 10, 100, and 1000 nM TCC. The results are expressed as fold change relative to the vehicle control (A) or to the T_3-induced levels (B) as described in the Materials and Methods section. Significance is indicated by an 'a' ($p \leq 0.05$ relative to control in "A" or relative to the T_3-induced response in "B", Wilcoxon). See Figure 1 legend for more graph details.

exposed treatments) with 1000 nM TCC showing a 1.5-fold reduction (p = 0.001, Wilcoxon) relative to vehicle control (part A of Figure 1).

The remaining four biopsies from each animal were exposed to 10 nM T_3 or 10 nM T_3 plus three concentrations of TCS (1, 10, and 100 nM), mTCS (1, 10, and 100 nM), or TCC (10, 100, and

1000 nM). This level of hormone alone increased *TRβ* transcript levels by 15-fold ± 3-fold (n = 41, data not shown) (mean = 9.6 ± 0.8, median = 7.8, n = 50) and reduced *RLKI* transcript levels by 3-fold ± 0.03-fold (n = 38, data not shown); results consistent with those reported previously.[29,30] Because we wished to determine whether TCS, mTCS, and TCC exposure altered TH-dependent responses of biopsies from each individual animal, we expressed the T_3/test chemical exposure data relative to the transcript levels obtained with T_3 alone (part B of Figure 1). In the presence of T_3, TCS, mTCS, and TCC did not significantly affect the steady-state levels of *TRβ* and *RLKI* transcript levels (part B of Figure 1).

HSP30 and *CAT* transcript levels were measured as indicators of cellular stress. Exposure to 1 and 10 nM TCS significantly increased the *HSP30* transcript levels relative to vehicle control by 4.5-fold (p = 0.010, Wilcoxon) and 3.7-fold (p = 0.050, Wilcoxon) respectively with an overall tendency toward significance over the concentration range (p = 0.074, Kendall's W) (part A of Figure 2). *CAT* transcript levels were increased upon exposure to TCS (p = 0.050, Kendall's W) with the 10 nM TCS increasing *CAT* levels by 2.4-fold (p = 0.026, Wilcoxon) (part A of Figure 2). *HSP30* transcript levels were significantly decreased with 10 nM mTCS treatment by 1.6-fold (p = 0.028, Wilcoxon) relative to vehicle control while *CAT* transcript levels were not significantly affected by mTCS (part A of Figure 2). TCC significantly increased the *HSP30* transcript levels (p = 0.0001, Kendell's W; p = 0.0001 for 1000 nM, Wilcoxon), whereas *CAT* transcript levels were increased at 1000 nM TCC by 1.29-fold (p = 0.042, Wilcoxon) (part A of Figure 2).

HSP30 transcript levels have been shown to increase when exposed to TH.[29,30,37] Treatment of biopsies with T_3 resulted in a 10 ± 4-fold (n = 41, data not shown) induction of *HSP30* transcript levels relative to the vehicle control, consistent with previously reported data.[29,30,37] TH induces oxidative stress and previous results have shown *CAT* transcript levels to decrease in response to TH treatment.[29,30,38] T_3 treatment alone resulted in a 1.4 ± 0.3-fold (n = 45, data not shown) decrease in *CAT* transcript levels relative to the vehicle control, which is consistent with previous reported observations.[29,30,38] TCS and mTCS did not have an effect on the T_3-induced levels of *HSP30* and *CAT* transcripts (part B of Figure 2). In contrast, *HSP30* transcripts levels were significantly increased upon exposure to TCC (p = 0.004, Kendall's W) with the increase occurring at 100 and 1000 nM TCC (7.5-fold, p = 0.028; 6.2-fold, p = 0.001, respectively; Wilcoxon) relative to T_3 treatment alone (part B of Figure 2). T_3-induced levels of *CAT* transcripts were not significantly changed by TCC treatment (part B of Figure 2).

Mammalian Cell Exposures. GH3 cells were exposed to each TCS, mTCS, and TCC in both the presence and absence of 10 nM T_3 to examine the effects that these chemicals have on the levels of *Gh*, *Dio1*, *Prl*, and *Hsp70* transcripts after 48 h of exposure. The concentrations chosen for this series of experiments were based upon the results obtained in the C-fin assays above and those obtained in a study on androgenic effects published in mammalian cells.[27] The median values for the *rpL8* normalizer transcript were highly comparable between treatments suggesting that RNA integrity was not adversely compromised by treatments (Table S3 of the Supporting Information). In the first experimental set, GH3 cells were exposed to 1−1000 nM TCS (Figure 3). Overall, significant differences were observed for *Gh*, *Dio1*, and *Hsp70* transcripts (p = 0.0001 for all transcripts comparing control to all TCS only treatments,

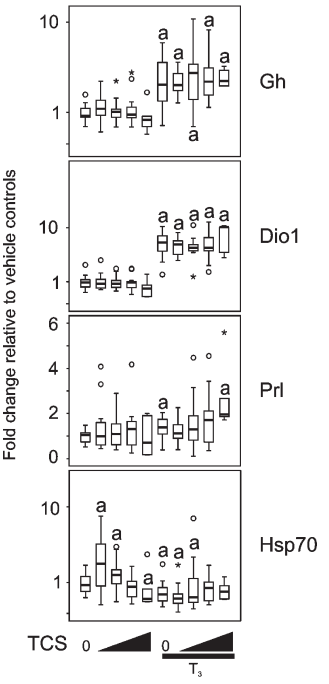


Figure 3. QPCR analysis of growth hormone (*Gh*), deiodinase I (*Dio1*), prolactin (*Prl*), and heat shock protein 70 (*Hsp70*) transcript levels in GH3 cells exposed to TCS. These cells (n = 17 wells for all treatment conditions with the exception of n = 5 for 1000 nM TCS in the absence and presence of T_3) were exposed to vehicle control (0) or 1, 10, 100, and 1000 nM TCS in the absence and presence of 10 nM T_3 for 48 h. The results are expressed as fold change relative to the vehicle control. Statistical significance of the mean relative fold change values compared to the vehicle control is denoted by 'a' ($p \leq 0.05$, MWU) and relative to T_3 treatment by 'b' ($p \leq 0.05$, MWU). See the Figure 1 legend for more graph details.

Kruskal−Wallis (KW)). The ∼3- and 6-fold increase in *Gh* and *Dio1* mRNAs relative to the vehicle control respectively upon exposure to T_3 (leftmost bar, Figure 3; p = 0.0001 for both; Mann−Whitney U (MWU)) was the sole contributor to the observed significance. Therefore, TCS did not alter the hormone-dependent induction of either transcript nor did it have an effect on basal levels in the absence of T_3 (Figure 3).

Prl transcripts have previously been shown to be increased or decreased by TH treatment depending upon the cell type.[34] Overall, *Prl* transcripts did not show a significant difference (p = 0.105, KW) (Figure 3). *Prl* transcript was a borderline responder to T_3 treatment, if responding at all. In most experiments, no significant alteration in *Prl* transcript levels were observed (compare Figures 3−5 and data not shown). Where a significant increase was observed, it was very modest at 1.4-fold (p = 0.011, MWU; Figure 3) relative to vehicle control. In the presence of T_3 treatment, 1000 nM TCS significantly increased *Prl* transcript levels relative to T_3 treatment alone by 2-fold (p = 0.006, MWU; Figure 3).

In contrast, exposure to TCS resulted in a significant alteration in *Hsp70* transcript levels (p = 0.0001, KW; Figure 3). This was due to multiple factors. First, TCS treatment alone increased *Hsp70* transcript levels (p = 0.007, KW for control and TCS only concentrations) at 1 and 10 nM relative to the vehicle control by 2.6-fold (p = 0.044, MWU) and 1.5-fold (p = 0.034, MWU), respectively. Exposure to 1000 nM TCS resulted in the slight

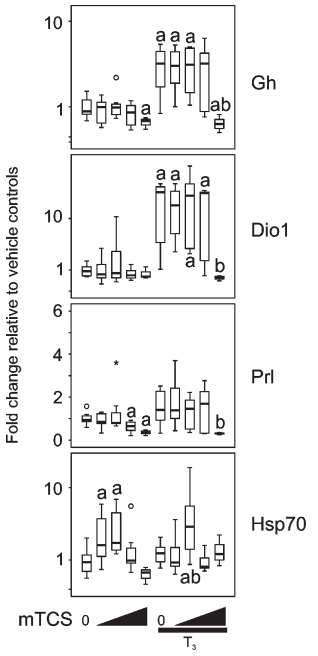


Figure 4. QPCR analysis of growth hormone (*Gh*), deiodinase I (*Dio1*), prolactin (*Prl*), and heat shock protein 70 (*Hsp70*) transcript levels in GH3 cells exposed to mTCS. These cells (n = 8 wells for all treatment conditions with the exception of n = 3 for 1000 nM mTCS in the absence and presence of T_3) were exposed to vehicle control (0) or 1, 10, 100, and 1000 nM mTCS in the absence and presence of 10 nM T_3 for 48 h. The results are expressed as fold change relative to the vehicle control. Statistical significance of the mean relative fold change values compared to the vehicle control is denoted by 'a' ($p \leq 0.05$, MWU) and relative to T_3 treatment by 'b' ($p \leq 0.05$, MWU). Further graph details are presented in the Figure 1 legend.

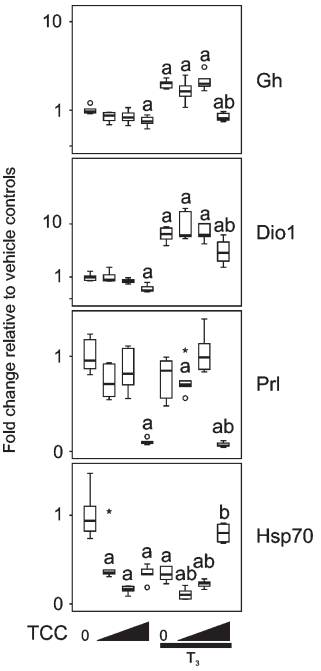


Figure 5. QPCR analysis of growth hormone (*Gh*), deiodinase I (*Dio1*), prolactin (*Prl*), and heat shock protein 70 (*Hsp70*) transcript levels in GH3 cells exposed to TCC. These cells (n = 6 wells per treatment condition) were exposed to vehicle control (0) or 10, 100, and 1000 nM TCC in the absence and presence of 10 nM T_3 for 48 h. The results are expressed as fold change relative to the vehicle control. Statistical significance of the mean relative fold change values compared to the vehicle control is denoted by 'a' ($p \leq 0.05$, MWU) and relative to T_3 treatment by 'b' ($p \leq 0.05$, MWU). Further graph details are presented in the Figure 1 legend.

reduction of *Hsp70* transcript levels relative to the vehicle control by 1.2-fold (p = 0.050, MWU; Figure 3).

T_3 treatment alone reduced the *Hsp70* transcript levels relative to vehicle control by 9.1-fold (p = 0.009, MWU), but this response to TH was largely unchanged, relative to TH treatment alone, when TCS was co-administered with TH (p = 0.393, KW for T_3 and T_3 plus TCS concentrations; Figure 3).

In the second experimental set, GH3 cells were exposed to 1−1000 nM mTCS (Figure 4). All four gene transcripts showed significant changes among all treatments (*Gh* p = 0.0001, *Dio1* p = 0.0001, *Prl* p = 0.013, *Hsp70* p = 0.001, KW, Figure 4). Closer examination of the data revealed that 1000 nM mTCS treatment significantly reduced *Gh* transcript levels by 2.1-fold (p = 0.025, MWU; Figure 4), whereas no effect of mTCS was observed on *Dio1* transcripts (Figure 4). In contrast, mTCS affected *Prl* and *Hsp70* mRNA abundance (p = 0.02 and 0.004, respectively; KW for control and TCS only concentrations). Specifically, 100 and 1000 nM mTCS reduced *Prl* transcript levels significantly relative to vehicle control by 1.6-fold (p = 0.016, MWU) and 2.8-fold (p = 0.014, MWU) (Figure 4) and *Hsp70* transcript levels were significantly increased by 1 and 10 nM mTCS by 2.8-fold (p = 0.046, MWU) and 3.3-fold (p = 0.005, MWU), respectively (Figure 4).

The response to T_3 alone, in the mTCS experiment, was similar to the TCS experimental set, for *Gh* and *Dio1* of transcripts with 3.6-fold (p = 0.006, MWU) and 19-fold (p = 0.004, MWU) increases, respectively (Figure 4). Analysis of the T_3 exposed set of cells showed both transcripts approaching significance (p = 0.093 and 0.061 respectively, KW). The major contributing factor was a marked reduction in the T_3-induced response of both transcripts (7.9-fold, p = 0.014; 35-fold, p = 0.014, respectively; MWU) when the cells were coexposed to 1000 nM mTCS (Figure 4).

The reduction in *Prl* transcript levels observed with mTCS exposure alone was also observed at the highest concentration tested in the presence of T_3 where 1000 nM mTCS significantly reduced the *Prl* transcript levels relative to T_3 treatment alone by 18-fold (p = 0.025, MWU) (Figure 4).

T_3 treatment alone did not significantly alter the *Hsp70* transcript levels (Figure 4). However, there was a significant effect of mTCS detected in the context of T_3 treatment (p = 0.015, KW) where exposure to 10 nM mTCS increased *Hsp70* transcript levels by 3.6-fold relative to T_3 treatment alone (p = 0.021, MWU) (Figure 4).

In the third experimental set, GH3 cells were exposed to 10−1000 nM TCC (Figure 5). TCC exposure resulted in significant changes in all four transcript abundances (p = 0.0001 for all, KW) (Figure 5). TCC significantly reduced *Gh* transcript levels relative to the vehicle control (p = 0.021, KW) with 1000 nM TCC resulting in a significant decrease relative to vehicle control by 1.6-fold (p = 0.004, MWU) (Figure 5). TCC significantly reduced *Dio1* transcript levels relative to the vehicle control (p = 0.002, KW) with 1000 nM TCC resulting in a significant decrease relative to vehicle control by 2.6-fold (p = 0.004, MWU) (Figure 5). Moreover, TCC significantly reduced

Prl transcript levels relative to the vehicle control (p = 0.001, KW) with 1000 nM TCC resulting in a significant decrease relative to vehicle control by 2.2-fold (p = 0.004, MWU) (Figure 5). Finally, the most marked response to TCC exposure was evident with the *Hsp70* transcripts (p = 0.0001, KW) where TCC reduced *Hsp70* transcript levels relative to vehicle control at 10, 100, and 1000 nM TCC by 2.2-fold (p = 0.025, MWU), 6.3-fold (p = 0.004, MWU), and 2.9-fold (p = 0.004, MWU), respectively (Figure 5).

Again, the response to T_3 was similar to Figures 3 and 4 for the *Gh* and *Dio1* transcripts (2.3-fold, p = 0.004; 7-fold, p = 0.006, MWU; Figure 5). *Prl* transcript abundance was unchanged, whereas T_3 treatment alone reduced the *Hsp70* transcript levels relative to vehicle control by 2.9-fold (p = 0.004, MWU) (Figure 5). It is evident that the *Prl* and *Hsp70* transcript responses to T_3 vary in comparing the three experimental sets. Additional experiments (data not shown) also confirmed this observation. The reason for this is not clear but may be related to the borderline responses typically observed.

Co-administration of T_3 and TCC resulted in significant changes in all four transcripts relative to T_3 alone (*Gh* p = 0.002, *Dio1* p = 0.037, *Prl* p = 0.001, *Hsp70* p = 0.0001, KW, Figure 5). *Gh*, *Dio1*, and *Prl* mRNAs showed lesser induction levels in the presence of 1000 nM TCC (3.1-fold, p = 0.004; 1.8-fold, p = 0.028; 11-fold, p = 0.004, respectively; MWU) (Figure 5).

TCC altered the *Hsp70* transcript levels at all concentrations tested when co-administered with T_3. At 10 and 100 nM TCC, *Hsp70* transcript levels were reduced relative to T_3 treatment alone by 3.9-fold (p = 0.004, MWU) and 1.5-fold (p = 0.037, MWU), respectively. At 1000 nM TCC, *Hsp70* levels were increased by 2.5-fold relative to T_3 treatment alone (p = 0.004, MWU) (Figure 5).

DISCUSSION

We showed previously that in vivo exposure of premetamorphic *Rana catesbeiana* tadpoles to TCS accelerated TH-dependent postembryonic development as indicated by hindlimb growth while reducing TH-induced *TRβ* transcript levels in the dorsal tail fin.[20] It is surprising that we did not find any interference with TH signaling pathways in either the tail biopsies or the cultured rat GH3 cells exposed to similar concentrations. The refractory nature of the GH3 cells may indicative of an indirect mode of action such as acting upon liver TH metabolism as was previously reported.[23−25] The lack of response from the tail biopsies was surprising because we had previously seen evidence of TH-disruptive effects on a cultured *Xenopus* line [20] suggesting a direct cellular response. It is possible that there may be species differences or that sensitivity may be influenced by cell or tissue type. At first glance, the C-fin results also suggest that perhaps an intact animal is required for the TH-disrupting effects of TCS.

However, the data from the biological metabolite, mTCS, suggests an alternate mechanism. Because mTCS affected all of the TH-responsive gene transcripts tested to some degree, this demonstrates that mTCS is capable of acting at a cellular level. Whole animals are exposed to TCS in nonsterile water where mTCS conversion is known to take place through bacterial action.[39] Thus, the previous effects observed by whole animal exposure could have been due to bacterial conversion. It is also possible that certain tissues or cells have the capacity to methylate TCS as mTCS has been found in fish.[19] However, the extent of contribution of these mechanisms is currently unknown[39] and needs to be examined further, particularly because mTCS is more persistent in the environment.[11]

We also found TCC disrupted the transcript levels of TH-responsive genes in the GH3 cells and in the tail fin biopsies. A modest effect was observed for *RLKI* transcript at the highest concentration (1000 nM) but no effect on *TRβ* transcript was observed in the frog tissue. The mammalian cells showed an effect in all three TH-responsive transcripts (*Gh*, *Dio1*, and *Prl*) again only at 1000 nM. It should be noted that it is possible that residual TH in the hormone-stripped serum-containing medium used for the cell culture may be contributing to the observed decrease in TH-responsive transcripts at high concentrations of mTCS and TCC alone. Overall, our results are consistent with previous work by others that imply that TCC acts on nuclear hormone receptors at high concentrations based upon reported effects of TCC on androgen-, estrogen-, and cortisone-mediated pathways.[27,41]

Another striking observation is that all three chemical treatments altered at least one indicator of cellular stress and these alterations were influenced by TH status. Stress is known to affect the rate of frog metamorphosis and alter tissue-responsiveness to TH.[40] TCS elicited a cellular stress response in the C-fin assay as indicated by the altered *CAT* and *HSP30* transcript levels. If whole animal exposure resulted in a stress response due to TCS exposure, this could possibly influence frog metamorphosis. Previous work using heat shock as a stressor showed that the extent of TH-induced *TRβ* transcript accumulation [40] was reduced in the tail; an observation that is consistent with the in vivo TCS exposure results. mTCS had a minimal effect on stress markers at the time point and this may be due to different induction kinetics. This possibility could be tested by examining transcript levels at additional time points.

Hsp70 transcript levels in the GH3 cells showed clearer concordance between TCS and mTCS responses, particularly at the lower concentrations tested (1 and 10 nM) in the absence of TH, suggesting that induction of a cellular stress response is a common mechanism. The apparent lack of response or even decrease at the higher concentrations may be related to the timing of the experiment (below).

Tadpole tail fin biopsies showed evidence of stress upon exposure to 1000 nM TCC. The remarkable reduction of *Hsp70* transcripts in the GH3 cells upon TCC exposure at all concentrations tested suggests the cellular capacity to respond to stress may have been adversely affected. The observed reduction may be the result of a negative feedback loop involving *Hsp70* induction. High levels of Hsp70 proteins can be detrimental to the cell.[42] *Hsp70* mRNA half-life is 1 h in cells after stress[43,44] and a parallel decrease in *Hsp70* mRNA levels was shown with an accumulation of Hsp70 protein levels. Hsp70 protein can bind *Hsp70* mRNA possibly as a mechanism to limit its protein expression and the decrease in mRNA levels is thought to be dependent on the Hsp70 protein levels.[45] Because the chemical exposure in the present study was 48 h in length, TCC could have caused a stress event and transiently increased *Hsp70* transcript levels. However, at the time point the transcript levels were measured, the *Hsp70* mRNA levels could have already decreased. Further work on an expanded time course and evaluation of Hsp70 protein levels would address this issue. Given the observed effects in cell and organ culture, examination of the effects of TCC on postembryonic development of frog tadpoles and on intact mammals is warranted.

■ ASSOCIATED CONTENT

Supporting Information. Chemical structures for T_3, TCS, mTCS, and TCC; mTCS synthesis and characterization, cell and organ culture methodology, RNA preparation, and QPCR and statistical analyses; list of the QPCR primers that are used in the present study to measure the mRNA steady-state levels of selected transcripts along with QPCR parameters and sequence information; comparison of Ct values obtained for rpL8 mRNAs in the C-fin assays and GH3 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (250) 721-6146; fax: (250) 721-8855; e-mail: chelbing@uvic.ca.

■ ACKNOWLEDGMENT

This work was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) to C.C.H. and J.E.W.

■ REFERENCES

(1) Bester, K. Triclosan in a sewage treatment process - balances and monitoring data. *Water Res.* **2003**, *37* (16), 3891–6.

(2) Sabaliunas, D.; Webb, S. F.; Hauk, A.; Jacob, M.; Eckhoff, W. S. Environmental fate of Triclosan in the River Aire Basin, UK. *Water Res.* **2003**, *37* (13), 3145–54.

(3) Tierno, P. M. Efficacy of triclosan. *Am. J. Infect. Control* **1999**, *27* (1), 71–2.

(4) Heath, R. J.; Li, J.; Roland, G. E.; Rock, C. O. Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. *J. Biol. Chem.* **2000**, *275* (7), 4654–9.

(5) McDonnell, G.; Pretzer, D. Action and targets of triclosan. *ASM News* **1998**, *64* (12), 670–1.

(6) McMurry, L. M.; Oethinger, M.; Levy, S. B. Triclosan targets lipid synthesis. *Nature* **1998**, *394* (6693), 531–2.

(7) Perencevich, E. N.; Wong, M. T.; Harris, A. D. National and regional assessment of the antibacterial soap market: A step toward determining the impact of prevalent antibacterial soaps. *Am. J. Infect. Control* **2001**, *29* (5), 281–3.

(8) Sapkota, A.; Heldler, J.; Halden, R. U. Detection of triclocarban and two co-contaminating chlorocarbanilides in US aquatic environments using isotope dilution liquid chromatography tandem mass spectrometry. *Environ. Res.* **2007**, *103* (1), 21–9.

(9) Halden, R. U.; Paull, D. H. Co-occurrence of triclocarban and triclosan in US water resources. *Environ. Sci. Technol.* **2005**, *39* (6), 1420–26.

(10) Koplin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton, H. T. Response to comment on "Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999−2000: A national reconnaissance. *Environ. Sci. Technol.* **2002**, *36* (18), 4004.

(11) Lindstrom, A.; Buerge, I. J.; Poiger, T.; Bergqvist, P. A.; Muller, M. D.; Buser, H. R. Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater. *Environ. Sci. Technol.* **2002**, *36* (11), 2322–29.

(12) Ying, G. G.; Yu, X. Y.; Kookana, R. S. Biological degradation of triclocarban and triclosan in a soil under aerobic and anaerobic conditions and comparison with environmental fate modelling. *Environ. Pollut.* **2007**, *150* (3), 300–5.

(13) Sandborgh-Englund, G.; Adolfsson-Erici, M.; Odham, G.; Ekstrand, J. Pharmacokinetics of triclosan following oral ingestion in humans. *J. Toxicol. Environ. Health A* **2006**, *69* (20), 1861–73.

(14) Scharpf, L. G., Jr.; Hill, I. D.; Maibach, H. I. Percutaneous penetration and disposition of triclocarban in man: body showering. *Arch. Environ. Health* **1975**, *30* (1), 7–14.

(15) Maibach, H. I.; Feldman, R. J.; Milby, T. H.; Serat, W. F. Regional variation in percutaneous penetration in man. Pesticides. *Arch. Environ. Health* **1971**, *23* (3), 208–11.

(16) Adolfsson-Erici, M.; Pettersson, M.; Parkkonen, J.; Sturve, J. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere* **2002**, *46* (9−10), 1485–9.

(17) Dayan, A. D. Risk assessment of triclosan [Irgasan (R)] in human breast milk. *Food Chem. Toxicol.* **2007**, *45* (1), 125–9.

(18) Wolff, M. S.; Teitelbaum, S. L.; Windham, G.; Pinney, S. M.; Britton, J. A.; Chelimo, C.; Godbold, J.; Biro, F.; Kushi, L. H.; Pfeiffer, C. M.; Calafat, A. M. Pilot study of urinary biomarkers of phytoestrogens, phthalates, and phenols in girls. *Environ. Health Perspect.* **2007**, *115* (1), 116–21.

(19) Balmer, M. E.; Poiger, T.; Droz, C.; Romanin, K.; Bergqvist, P. A.; Muller, M. D.; Buser, H. R. Occurrence of methyl triclosan, a transformation product of the bactericide triclosan, in fish from various lakes in Switzerland. *Environ. Sci. Technol.* **2004**, *38* (2), 390–5.

(20) Veldhoen, N.; Skirrow, R.; Osachoff, H.; Wigmore, H.; Clapson, D.; Gunderson, M.; van Aggelen, G.; Helbing, C. The bactericidal agent Triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquat. Toxicol.* **2006**, *80*, 217–27.

(21) Fort, D. J.; Rogers, R. L.; Gorsuch, J. W.; Navarro, L. T.; Peter, R.; Plautz, J. R. Triclosan and anuran metamorphosis: no effect on thyroid-mediated metamorphosis in *Xenopus laevis*. *Toxicol. Sci.* **2010**, *113* (2), 392–400.

(22) Helbing, C.; van Aggelen, G.; Veldhoen, N. Triclosan affects thyroid hormone-dependent metamorphosis in anurans. *Toxicol. Sci.* **2010**, *119*, 417–8.

(23) Paul, K. B.; Hedge, J. M.; DeVito, M. J.; Crofton, K. M. Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in Young Long-Evans rats. *Toxicol. Sci.* **2010**, *113*, 367–79.

(24) Paul, K. B.; Hedge, J. M.; DeVito, M. J.; Crofton, K. M. Developmental triclosan exposure decreases maternal and neonatal thyroxine in rats. *Environ. Toxicol. Chem.* **2010**, *29*, 2840–4.

(25) Zorrilla, L. M.; Gibson, E. K.; Jeffay, S. C.; Crofton, K. M.; Setzer, W. R.; Cooper, R. L.; Stoker, T. E. The effects of Triclosan on puberty and thyroid hormones in male Wistar rats. *Toxicol. Sci.* **2009**, *107*, 56–64.

(26) Crofton, K. M. Thyroid disrupting chemicals: mechanisms and mixtures. *Int. J. Androl.* **2008**, *31*, 209–22.

(27) Chen, J. G.; Ahn, K. C.; Gee, N. A.; Ahmed, M. I.; Duleba, A. J.; Zhao, L.; Gee, S. J.; Hammock, B. D.; Lasley, B. L. Triclocarban enhances testosterone action: A new type of endocrine disruptor?. *Endocrinol.* **2008**, *149*, 1173–9.

(28) Foran, C. M.; Bennett, E. R.; Benson, W. H. Developmental evaluation of a potential non-steroidal estrogen: triclosan. *Mar. Environ. Res.* **2000**, *50*, 153–6.

(29) Hinther, A.; Domanski, D.; Vawda, S.; Helbing, C. C. C-fin: A cultured frog tadpole tail fin biopsy approach for detection of thyroid hormone-disrupting chemicals. *Environ. Toxicol. Chem.* **2010**, *29*, 380–8.

(30) Hinther, A.; Vawda, S.; Skirrow, R. C.; Veldhoen, N.; Collins, P.; Cullen, J. T.; van Aggelen, G.; Helbing, C. C. Nanometals induce stress and alter thyroid hormone action in Amphibia at or below North American water quality guidelines. *Environ. Sci. Technol.* **2010**, *44*, 8314–21.

(31) Domanski, D.; Helbing, C. C. Analysis of the *Rana catesbeiana* tadpole tail fin proteome and phosphoproteome during T_3-induced apoptosis: identification of a novel type I keratin. *BMC Devel. Biol.* **2007**, *7*, 94.

(32) Spindler, S. R.; Mellon, S. H.; Baxter, J. D. Growth hormone gene transcription is regulated by thyroid and glucocorticoid hormones in cultured rat pituitary tumor cells. *J. Biol. Chem.* **1982**, *257*, 11627–32.

(33) Davey, J. C.; Nomikos, A. P.; Wungjiranirun, M.; Sherman, J. R.; Ingram, L.; Batki, C.; Lariviere, J. P.; Hamilton, J. W. Arsenic as an endocrine disruptor: arsenic disrupts retinoic acid receptor-and thyroid hormone receptor-mediated gene regulation and thyroid hormone-mediated amphibian tail metamorphosis. *Environ. Health Perspect.* **2008**, *116*, 165–72.

(34) Forman, B. M.; Yang, C. R.; Stanley, F.; Casanova, J.; Samuels, H. H. c-erbA protooncogenes mediate thyroid hormone-dependent and independent regulation of the rat growth hormone and prolactin genes. *Mol. Endocrinol.* **1988**, *2*, 902–11.

(35) Williams, G. T.; Morimoto, R. I. Maximal stress-induced transcription from the human HSP70 promoter requires interactions with the basal promoter elements independent of rotational alignment. *Mol. Cell. Biol.* **1990**, *10*, 3125–36.

(36) Taylor, A. C.; Kollros, J. J. Stages in the normal development of Rana pipiens larvae. *Anat. Record* **1946**, *94*, 7–24.

(37) Helbing, C.; Gallimore, C.; Atkinson, B. G. Characterization of a *Rana catesbeiana* hsp30 gene and its expression in the liver of this amphibian during both spontaneous and thyroid hormone-induced metamorphosis. *Devel. Genet.* **1996**, *18*, 223–33.

(38) Kashiwagi, A.; Hanada, H.; Yabuki, M.; Kanno, T.; Ishisaka, R.; Sasaki, J.; Inoue, M.; Utsumi, K. Thyroxine enhancement and the role of reactive oxygen species in tadpole tail apoptosis. *Free Radic. Biol. Med.* **1999**, *26*, 1001–9.

(39) Chen, X.; Nielsen, J. L.; Furgal, K.; Liu, Y.; Lolas, I. B.; Bester, K. Biodegradation of triclosan and formation of methyl-triclosan in activated sludge under aerobic conditions. *Chemosphere* **2011**, 10.1016/j.chemosphere.2011.03.042.

(40) Helbing, C. C.; Atkinson, B. G. 3,5,3′-Triiodothyronine-induced carbamyl phosphate synthetase gene expression is stabilized in the liver of Rana catesbeiana tadpoles during heat shock. *J. Biol. Chem.* **1994**, *269*, 11743–50.

(41) Chen, J.; Ahn, K. C.; Gee, N. A.; Gee, S. J.; Hammock, B. D.; Lasley, B. L. Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products. *Toxicol. Appl. Pharmacol.* **2007**, *221*, 278–84.

(42) Arispe, N. J.; Simakova, O.; Doh, M.; Diaz, J.; De Maio, A. Toxic extracellular interaction of heat shock proteins with cells is mediated by phosphatidylserine. *Biophys. J.* **2004**, *86* (1), 90a.

(43) Theodorakis, N. G.; Morimoto, R. I. Posttranscriptional regulation of Hsp70 expression in human-cells - effects of heat-shock, inhibition of protein-synthesis, and adenovirus infection on translation and messenger-RNA stability. *Mol. Cell. Biol.* **1987**, *7*, 4357–4368.

(44) De Maio, A. Heat shock proteins. Facts, thoughts, and dreams. Reply. *Shock* **1999**, *12*, 324–325.

(45) Balakrishnan, K.; De Maio, A. Heat shock protein 70 binds its own messenger ribonucleic acid as part of a gene expression self-limiting mechanism. *Cell Stress Chaperone* **2006**, *11*, 44–50.