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Reverse genetics in the tide pool: knock-down of target gene expression via RNA interference in the copepod *Tigriopus californicus*

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Abstract

Reverse genetic tools are essential for characterizing phenotypes of novel genes and testing functional hypotheses generated from next-generation sequencing studies. RNA interference (RNAi) has been a widely used technique for describing or quantifying physiological, developmental or behavioural roles of target genes by suppressing their expression. The marine intertidal copepod *Tigriopus californicus* has become an emerging model for evolutionary and physiological studies, but this species is not amenable to most genetic manipulation approaches. As crustaceans are susceptible to RNAi-mediated gene knock-down, we developed a simple method for delivery of gene-specific double-stranded RNA that results in significant suppression of target gene transcription levels. The protocol was examined on five genes of interest, and for each, at least 50% knock-down in expression was achieved. While knock-down levels did not reach 100% in any trial, a well-controlled experiment with one heat-shock gene showed unambiguously that such partial gene suppression may cause dramatic changes in phenotype. Copepods with suppressed expression of heat-shock protein beta 1 (*hspb1*) exhibited dramatically decreased tolerance to high temperatures, validating the importance of this gene during thermal stress, as proposed by a previous study. The application of this RNAi protocol in *T. californicus* will be invaluable for examining the role of genes putatively involved in reproductive isolation, mitochondrial function and local adaptation.

Keywords: copepod, gene suppression, heat-shock protein, RNA interference

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Introduction

The advent of next-generation sequencing has allowed genome-level questions to be addressed in virtually any organism, and this revolution has been particularly important for studies of nonmodel organisms (Wang *et al.* 2009; Wheat 2010). In a single transcriptome-sequencing study (RNA-seq), for instance, researchers can now obtain the DNA sequence and expression levels of thousands of protein-coding genes (Mortazavi *et al.* 2008; Wilhelm & Landry 2009; Yassour *et al.* 2009). Studies utilizing these data are hence able to screen the entire genome and identify candidate regions that may be functionally involved in physiological, developmental or evolutionary processes. While this approach has been successfully applied to countless nonmodel species, very few of these possess molecular tools that can be used in subsequent experiments aimed at confirming the relevance or characterizing the function of candidate genes.

The intertidal copepod *Tigriopus californicus* is an emerging model for research in marine physiology (Burton & Feldman 1982; Goolish & Burton 1988; Willett & Burton 2002), neurobiology (Andrew *et al.* 2012), ecology (Dethier 1980; Powlik 1998) and evolution (Burton & Lee 1994; Edmands 1999; Voordouw & Anholt 2002). This species inhabits high intertidal splash pools along the west coast of North America, from Baja California, Mexico, to Alaska, and it is a highly tractable experimental system. Laboratory hybrids that are formed from crossing isolated and divergent populations consistently exhibit 'breakdown' in several life-history and metabolic traits (Burton 1990; Edmands 1999; Ellison & Burton 2006), which has made *T. californicus* an excellent model for examining genetic incompatibilities that arise during allopatric differentiation and speciation (Burton *et al.* 2006). Geographic isolation has also resulted in physiological adaptation to local environments, with isolated populations showing

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striking differences in tolerance to acute thermal stress (Willett 2010; Kelly *et al.* 2012; Pereira *et al.* 2014). These evolutionary patterns have motivated the use of RNA-seq to examine protein sequence evolution (Barreto *et al.* 2011; Barreto & Burton 2013) as well as gene expression response to heat stress (Schoville *et al.* 2012) in *T. californicus*. Results of these studies have highlighted several genes that potentially underlie the examined phenotypes. However, without a direct way to assess the role of individual genes, genome and transcriptome-sequencing experiments are largely limited to generating lists of candidate genes.

Here, we demonstrate the initial development of RNA interference (RNAi) methodology for knock-down of target genes in *T. californicus*. RNAi is a nucleic acid-based approach for post-transcriptional gene suppression and hence can be used to assess individual gene function and its relevance within target cellular pathways (Guo & Kemphues 1995; Fire *et al.* 1998). The phenomenon was discovered in *Caenorhabditis elegans* (Guo & Kemphues 1995; Fire *et al.* 1998), but has been shown to be evolutionarily conserved across eukaryotes (Cerutti & Casas-Mollano 2006; Shabalina & Koonin 2008). While this method is still more prominent in well-established genetic models like *C. elegans*, mice and *Drosophila*, it has been effectively used for studies in a wide range of taxonomic groups, including cnidarians (Lohmann *et al.* 1999; Dunn *et al.* 2007; Duffy *et al.* 2010; Fuchs *et al.* 2014), sponges (Rivera *et al.* 2011), flatworms (Orii *et al.* 2003; McGonigle *et al.* 2008; Sripa *et al.* 2011; Piratae *et al.* 2012), rotifers (Shearer & Snell 2007; Snell *et al.* 2011), tardigrades (Tenlen *et al.* 2013), gastropods (Knight *et al.* 2011) and arachnids (Schoppmeier & Damen 2001; Khila & Grbic 2007; Karim *et al.* 2010).

Briefly, RNAi is triggered by the delivery of gene-specific double-stranded RNA (dsRNA) to the cytoplasm. The dsRNA is cleaved into fragments of 21–25 nucleotides (small interfering RNAs, or siRNAs), which become associated with the multiprotein RNA-induced silencing complex (RISC). Based on sequence similarity, the RISC-siRNA structure then targets and degrades endogenous mRNAs before they can be used for translation into protein (Carthew 2001; Grishok *et al.* 2001; Grishok & Mello 2002; Hannon 2002).

A major obstacle in the development of an RNAi protocol is in achieving efficient dsRNA delivery into cells (Lendner *et al.* 2008; Britton *et al.* 2012; Sagi *et al.* 2013). Microinjection has been the most widely used method for in vivo delivery in arthropods (Bellés 2010; Terenius *et al.* 2011), including crustaceans (Liubicich *et al.* 2009; Kato *et al.* 2011; Sagi *et al.* 2013; King *et al.* 2014). In copepods, RNAi experiments have been performed via microinjection (Campbell *et al.* 2009; Dalvin *et al.* 2009) or by soaking in sea water containing dsRNA (Campbell *et al.* 2009; Carpio *et al.* 2011). Due to its minute size, *T. californicus* (cephalothorax length < 0.5 mm, Edmands & Harrison 2003) is not amenable to microinjection; besides high mortality from manipulation, population-level studies would be prohibitively laborious. Here, we report on attempts to perform RNAi in *T. californicus* using a variety of methods, and we show that electroporation is a viable method of dsRNA delivery for target gene knock-down in this copepod species. We present our most successful protocol by showing the transcriptional suppression and subsequent phenotypic effect of *hspb1*, a heat-shock protein gene that is putatively important in thermal tolerance in this species (Schoville *et al.* 2012), and also test its use on four additional genes of interest.

Materials and methods

Collection and culture of copepods

Copepods were collected from high intertidal rocky pools in San Diego (SD: 32°45′N, 117°15′W), California, and maintained in large populations in multiple 400-mL beakers filled with 0.2 μm filtered sea water (with salinity of 35 parts per thousand). Cultures were kept in incubators at 20 °C with a 12-h light:dark cycle and fed powdered *Spirulina* wafers. Beakers were mixed periodically to maintain outbreeding. Copepods were allowed to acclimate to laboratory conditions and were only used in experiments after a minimum of three generations in culture. *Tigriopus californicus* individuals achieve sexual maturity in 14–23 days after hatching. Copepods used in our RNAi experiments were kept in normal cultures in large populations until use. Only mature individuals of both sexes were used. The exact age of selected copepods was not known, but we do not expect this to strongly affect RNAi, as they can live and reproduce for several weeks to months after maturity. Males can mate continuously throughout their lives, while females mate only once yet can continue to lay clutches (by means of sperm storage) for at least several weeks after copulation. No attempts were made to deliver dsRNA in eggs or larvae in this study.

Gene selection and dsRNA synthesis

To develop an RNAi protocol for *T. californicus*, we sought a gene with detectable constitutive expression and potentially obvious phenotypic effect. Inducible heat-shock protein genes (*hsps*) are excellent candidates because they are the primary molecular chaperones during acute organismal stress (Feder & Hofmann 1999); in arthropods, knock-down of single *hsp* genes results in high mortality rates during physiological stress (Rinehart

et al. 2007; Benoit *et al*. 2010; Smith *et al*. 2012). We chose hsp beta-1 (*hspb1*), which was shown by Schoville *et al*. (2012) to have >100-fold upregulation during acute heat stress.

We used the gene sequence from Schoville *et al*. (2012) (GenBank Accession no. JW506233) to design PCR primers and then amplify a 385-bp region of *hspb1* (Table 1). The PCR primers were synthesized with the T7 promoter sequence (TAATACGACTCACTATAGGG) appended to their 5′ ends so that the T7 sequence is added to each strand of the amplicon. Before PCR, total RNA was isolated from a pool of ~100 copepods using the Tri-Reagent (Sigma) protocol, with tissue homogenization performed using zirconia/silica beads (0.5 mm diameter) in a Mini-Beadbeater (BioSpec). Residual DNA was removed by digestion with Turbo DNase (Ambion), and cDNA was synthesized from 2 μg RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems) in 20-μL reactions. Finally, PCR was performed in 25-μL reactions containing 1× PCR buffer (Promega GoTaq Flexi), 0.2 mM of each dNTP, 0.8 μM of each primer, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Promega) and 1 μL cDNA (equivalent to 50 ng RNA). Thermal cycling parameters consisted of an initial denaturation at 95 °C for 3 min, followed by 34 cycles of 95 °C for 20 s, 58 °C for 1 min, and 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Molecular weight of the PCR product was then checked via agarose electrophoresis.

To control for the possibility of nonspecific effects triggered by foreign nucleic acids, we generated a negative control dsRNA with sequence not matching any *T. californicus* gene. We designed T7-flanked primers for and amplified a 392-bp region of the pCR4-TOPO plasmid (Invitrogen) (forward primer: 5′-GTGCACGTCTG CTGTCAGAT-3′; reverse primer: 5′-CCATAAAACCGC CCAGTCTA-3′). This dsRNA was used as control dsRNA in some experiments, in addition to untreated controls (no dsRNA).

Eight microlitres of each T7-flanked PCR product was used as template for *in vitro* transcription using the MEGAscript Kit (Ambion), following manufacturer's protocol and with overnight incubation. The transcription product was then treated with Turbo DNase (37 °C for 1 h) and purified with an RNeasy column (Qiagen). Because both PCR primers had T7 sequences, both strands of the PCR product also contained T7 promoters; hence, no annealing step is generally necessary to generate dsRNA. Aliquots of the PCR and T7 transcription products were run side by side on a 2% agarose gel to confirm successful dsRNA synthesis (in a nondenaturing gel, dsRNA migrates slower than dsDNA of same size). Concentration and purity of dsRNA were assessed spectrophotometrically, and samples were stored at −80 °C until use.

Feeding of chitosan/dsRNA complexes

A potentially high-throughput and nonlethal method of dsRNA delivery is by simple feeding. We used the method developed by Zhang *et al*. (2010) to successfully deliver dsRNA and suppress gene expression in mosquito larvae (*Anopheles gambiae*). This approach utilizes the biopolymer chitosan for encapsulation, protection and delivery of nucleic acids (Mao *et al*. 2010; Rudzinski & Aminabhavi 2010). We followed the protocol in

Table 1 Primer sequences and features for *Tigriopus californicus* genes examined

Gene	GenBank accession	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Efficiency
For dsRNA*					
hsp beta-1	JW506233	ACAGCGGAGGAGTTCTTCAA	GGTGCCGTAATGGTCAACTT	385	NA
hsp70	JW519350	CTTTGTCGTTGGTGATGGTG	ATATCGTTCTGGTGGGTGGA	525	NA
serpin	JW510831	GTCTGCGGAGATAACGGAAA	TCACAACGAGAGCAATCGAC	188	NA
mtRPOL	DQ290240	CAAACCGTTAGGTCCCAAGGGC	GAGGCCTCGCCCAATTTGTCGC	604	NA
mRPS29	JW501954	AACCTGGGCGATATGATCTG	TGGAGTCATGCTTGAGCAAC	426	NA
For qPCR					
hsp beta-1	JW506233	CGATTTTCATCTGGGTCTCAA	TTGAAGAACTCCTCCGCTGT	175	1.97
hsp70	JW519350	CTGGATTGATGCTCTTGTTCA	CTCTGTGCCGACCTTTTCC	184	2.02
serpin	JW510831	AGAATCTCCCTCTGATTTGC	TCACCGCAAGGGCATTCACC	152	1.98
mtRPOL	DQ290240	GTCCTTTGGGTGTGATCTGG	GGTAGTCTGGGACCCTCCTC	134	1.94
mRPS29	JW501954	ACGATCCTTTGCACCATGAC	GTGCTCCTGGAGAGCTTGAC	149	1.95
GAPDH	JW506006	GGAGGAGGGGATGATGTTTT	CAACCACGAGCAATACGAGA	226	1.96
myosin	JW508385	GTGTCGCAAAAGCAAATGAC	GAACCTCAACCTCCTCCTCA	154	1.98

Primers used in PCR destined for dsRNA synthesis had a T7 promoter sequence (TAATACGACTCACTATAGGG) appended to their 5′ ends.

Zhang *et al.* (2010) closely. Briefly, a 0.02% (weight/volume) solution of chitosan (Sigma-Aldrich cat. no. C3646) was prepared by dissolving the polymer in sodium acetate buffer (0.1 M sodium acetate, 0.1 M acetic acid, pH 4.5). *Hspb1* dsRNA (32 μg) was diluted in 100 μL of 50 mM sodium sulphate, and this mixture was added to 100 μL chitosan solution. The mixture was heated to 55 °C for 1 min and then immediately vortexed at full speed for 30 s. After cooling to room temperature for 10 min, chitosan/dsRNA complexes were formed. The nanoparticles were pelleted by centrifugation (13 000 *g* for 10 min) and most of the supernatant removed, leaving the pellet in approximately 25 μL of solution. Copepod food (4–6 mg of ground *Spirulina* wafers) was mixed with the pellet by pipetting, and the mixture of food and nanoparticles was coated with 30 μL of pre-melted agarose (2% at 55 °C). The solidified plug was cut into three equal pieces (each containing approximately 10 μg entrapped dsRNA), and each piece was placed in a petri dish containing 25–30 adult copepods in 15 mL filtered seawater and kept in 20 °C incubator. Control samples were prepared in the same manner, but deionized H_2O used instead of dsRNA. Each trial contained three untreated control (no dsRNA) and three *hspb1* replicates, and transcription levels assessed by quantitative PCR (qPCR) at days 3 and 6 after feeding started.

Lipofection

We also used lipid-based carriers to deliver dsRNA to copepod cells, as this approach results in robust RNAi in animal cell lines (Felgner *et al.* 1987) and was successfully used in rotifers (Shearer & Snell 2007; Snell *et al.* 2011). Transfection solution was prepared by mixing 5 μL Lipofectamine RNAiMAX (Invitrogen) with 45 μL phosphate-buffered saline (PBS: 2 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl), and this solution transferred to another tube containing 47 μL PBS and 3 μL *hspb1* dsRNA (or deionized H_2O for untreated controls). The two solutions were mixed well by pipetting and allowed to incubate at room temperature for 30 min to form liposome/dsRNA complexes. The 100-μL transfection solution was then mixed with 100 μL of 2× artificial sea water (ASW, 1× salinity = 34 ppt) made with DEPC-treated molecular grade water (DEPC-H_2O) and transferred to a 1.5-mL centrifuge tube containing 25–30 adult copepods. Samples were incubated at 20 °C for 48 h, and copepods then transferred to petri dishes containing filtered sea water with food. Gene knock-down was assessed by qPCR 24 h later. We performed the experiment six times (2–3 control and test samples per trial), with varying dsRNA quantities (Table 2).

Delivery of dsRNA by rehydration

Lopez-Martinez *et al.* (2012) obtained strong target gene suppression in the aquatic larvae of the mosquito *Culex pipiens* using a simple rehydration method for dsRNA delivery. We modified their protocol for *T. californicus*. For each replicate, a group of 10–15 adult copepods was first dehydrated by soaking in 100 μL of 3× ASW for 30–60 min in a 1.5-mL centrifuge tube. This salinity level and soaking time were established in preliminary trials, with these two parameters adjusted until copepods became dehydrated (i.e. had shrunk in size and were immobile on bottom of the tube) but recovered once placed back in 1× ASW. After dehydration, the water was completely removed by pipetting and copepods resuspended in 30 μL 1× ASW containing 20–35 μg *hspb1* dsRNA (or no dsRNA for controls). Copepods were allowed to soak for 3–16 h at 20 °C, during which time osmotic balance was restored, possibly with dsRNA uptake as a result of rehydration. Copepods that recovered were then transferred to dishes containing filtered seawater and food at 20 °C, and gene expressions levels assessed 3 days after treatment. Each trial comprised 2–4 control and *hspb1* replicates (Table 2).

Electroporation for dsRNA delivery

Following protocols developed for in vivo delivery of nucleic acids in whole small animals via electroporation (Lohmann *et al.* 1999; Correnti *et al.* 2005; Zhao *et al.* 2008; Karim *et al.* 2010; Vierra & Irvine 2012; Fuchs *et al.* 2014), we tried a range of voltages (25–320 V), time constants (100 μs to 80 ms) and dsRNA concentrations (100 ng/μL to 1 μg/μL). For each replicate, a pool of 15–60 adult copepods were first rinsed in ASW (salinity 34 ppt), then transferred in 200 μL ASW to a 1.5-mL microcentrifuge tube and kept on ice until copepods became dormant on the bottom of the tube. All water from the tube was then pipetted out, and the copepods were resuspended in 100 μL of assay mixture. The assay mixture was composed of 70 μL ASW and 30 μL DEPC-H_2O containing either dsRNA (target or control) or no dsRNA (untreated control). Using a Pasteur pipette, the mixture containing copepods was carefully transferred to the bottom of a 0.4-cm electroporation cuvette (Bio-Rad), and the sample was electroporated in a Gene Pulser Xcell (Bio-Rad) electroporator with square-wave pulses. The full list of experiments and parameter combinations can be found in Table 2. After electroporation, the sample was kept at room temperature for 10 min before being transferred to a petri dish containing 15 mL filtered sea water with food and kept in 20 °C incubator until assessment of gene expression 3–5 days later.

Table 2 Summary of transfection experiments for RNA interference in *Tigriopus californicus*

Method	No. trials*	μg dsRNA in treatment	Parameters	Total no. control replicates	Total no. *hspb1* dsRNA replicates	% mortality in controls†	% *hspb1* knockdown‡
Feeding	5	10	Fed for 3 days	15	15	0–12	0
Lipofection	3	1	Incubated for 48 h	9	9	25–40	15–25
Lipofection	1	9	Incubated for 48 h	3	3	16–43	0–21
Lipofection	2	25	Incubated for 48 h	4	4	28–80	0–28
Rehydration	2	20	3× artificial sea water (ASW)/30 min → 1× ASW/3 h	6	6	0–10	0
Rehydration	2	35	3× ASW/60 min → 1× ASW/5 h	4	4	0–15	0
Rehydration	2	35	3× ASW/30 min → 1× ASW/16 h	6	8	0–18	0
Electroporation	1	23	1 pulse: 320 V–0.3 ms → 3 pulses: 25 V–10 ms	3	3	38–65	0
Electroporation	2	10	1 pulse: 250 V–0.1 ms	4	4	15–23	0
Electroporation	1	10	1 pulse: 250 V–1 ms	3	3	57–77	0
Electroporation	1	20	1 pulse: 125 V–20 ms	3	3	20–45	0
Electroporation	2	30	1 pulse: 125 V–20 ms	4	4	25–40	0–30
Electroporation	2	35	1 pulse: 125 V–20 ms	4	4	50–60	20–25
Electroporation	2	50	1 pulse: 125 V–20 ms	5	5	20–77	0–48
Electroporation	2	25	5 pulses: 100 V–2 ms	6	6	12–45	15–32
Electroporation	1	40	1 pulse: 100 V–20 ms	2	3	25–60	0
Electroporation	1	60	1 pulse: 100 V–20 ms	2	2	8–21	0–21
Electroporation	1	25	8 pulses: 80 V–20 ms	3	2	21–36	0–28
Electroporation	1	52	5 pulses: 50 V–10 ms	2	2	6–18	0–32
Electroporation	1	15	10 pulses: 50 V–20 ms	3	3	12–19	46–51
Electroporation	1	25	10 pulses: 50 V–20 ms	2	3	20–30	72–94
Electroporation	1	35	10 pulses: 50 V–20 ms	2	2	10–20	40–65
Electroporation	2	45	10 pulses: 50 V–20 ms	6	4	42–25	28–38
Electroporation	4	50	10 pulses: 50 V–20 ms	8	8	9–31	20–58
Electroporation	3	100	10 pulses: 50 V–20 ms	10	12	5–42	0–72
Electroporation	1	60	1 pulse: 50 V–80 ms	2	2	5–12	0–24
Electroporation	1	23	10 pulses: 50 V–80 ms	3	2	18–30	41–44
Electroporation	2	52	10 pulses: 25 V–10 ms	4	4	12–18	0–45
Electroporation	2	70	1 pulse: 100 V–5 ms → 10 pulses: 50 V–20 ms	5	6	18–27	52–94
Electroporation	1	50	1 pulse: 100 V–5 ms → 10 pulses: 50 V–20 ms	3	3	22–45	71–95
Electroporation	1	30	1 pulse: 100 V–5 ms → 10 pulses: 50 V–20 ms	4	4	19–40	42–93

*Total number of independent trials of each method with each parameter combination. Each trial included 2–4 replicates each of control (no dsRNA) and *hspb1* dsRNA treatments.

†Range of mortality levels observed across all control replicates, calculated as the proportion of individual copepods dead as a result of experimental manipulation.

‡Range of *hspb1* expression of replicates treated with *hspb1* dsRNA relative to controls of the respective experiment. % knockdown = (1 − expression relative to control) × 100.

Time-course of hspb1 *suppression*

After experimenting with 31 different combinations of technical parameters, we chose the most successful method, based on repeatability of significant *hspb1* knock-down and low manipulation-induced mortality. This involved electroporation with two sets of square-wave pulses: one pulse of 100 V for 5 ms, followed immediately by 10 pulses of 50 V for 20 ms (with 1 s interval between pulses) (Table 2). All subsequent experiments employed this procedure.

We first assessed the time of onset and short-term duration of *hspb1* knockdown. Five replicates of each of three treatments were electroporated: untreated control (no dsRNA), control dsRNA and target dsRNA (*hspb1*). Each replicate contained 60 adult copepods. Copepods were treated and maintained as above, and dead individuals were discarded daily. At days 2, 4, 6 and 8 after

electroporation, 10–15 copepods were sampled from each replicate and their RNA was isolated and DNase-treated as above. Relative expression of *hspb1* in each replicate was assessed by quantitative PCR, and an ANOVA was used to compare treatment groups. Following the ANOVA, Tukey's post hoc tests were used to compare changes in knock-down among sampling days.

While the control dsRNA allowed us to test for the possible affect of introducing a foreign nucleic acid into cells, we also verified that the target dsRNA (*hspb1*) did not trigger 'generalized' suppression of nontarget genes. Therefore, we monitored the relative expression of two other *T. californicus* genes (*hsp70* and *serpin*; Table 1) and tested whether they were affected by *hspb1* dsRNA treatment.

Thermotolerance during hspb1 suppression

After determining the time-course of *hspb1* knockdown, we performed another RNAi experiment to test the hypothesis that *hspb1* upregulation is essential for tolerance to acute heat stress in *T. californicus* from San Diego (Schoville *et al.* 2012). New samples containing 50 copepods each were subjected to electroporation under one of the three experimental treatments: *hspb1* dsRNA ($n = 5$), control dsRNA ($n = 3$) or no dsRNA ($n = 3$). After electroporation, samples were maintained in 20 °C incubator in petri dishes with food, monitored daily, and dead individuals discarded. Four days after electroporation, 10–15 individuals from each replicate were transferred to 1.5-mL tubes containing Tri-Reagent, and total RNA was isolated as above. These subsamples were used in qPCR to verify levels of *hspb1* expression in each replicate. The remaining 25–35 copepods in each replicate were then subjected to high thermal stress by exposing them to a semilethal temperature (36 °C, Willett 2010) in 15-mL tubes immersed in a water bath for 1 h. After the high temperature treatment, tubes were then immersed in a 20 °C water bath for recovery, and copepods were finally returned to their petri dishes. The exact number of individuals in each replicate was noted before heat stress, and the number of surviving individuals was assessed daily for 5 days after heat stress. The experiment was performed twice in its entirety, with new copepods, and all data were combined. The Kaplan–Meier method was applied to the survival data, and log-rank tests used to compare curves among treatment groups.

RNAi of additional genes

After achieving consistent knock-down of *hspb1* expression with electroporation, we tested our method on four additional genes of interest in *T. californicus*. We chose another highly inducible heat-shock protein (*hsp70*) from Schoville *et al.* (2012). As *T. californicus* is a model for studying nuclear–mitochondrial interactions (Burton *et al.* 2006), we targeted two nuclear-encoded genes previously hypothesized to be involved in hybrid dysfunction, namely the mitochondrial RNA polymerase (*mtRPOL*) and the mitochondrial ribosomal protein S29 (*mRPS29*). Finally, we are interested in examining the transcriptional landscape of *T. californicus* during osmotic stress. A recent result (Luke *et al.* 2007) highlighted the potential relevance of serine protease inhibitors (*serpin*) for osmoregulation during salinity stress, which is a common stress in *T. californicus* habitats (Burton & Feldman 1982; Goolish & Burton 1988). In general, these genes were selected because they may become the focus of future functional studies in *T. californicus*.

T7-flanked primers were designed from sequences generated by Schoville *et al.* (2012) (Table 1), and PCR and dsRNA synthesis were performed as detailed for *hspb1* above. For each gene, we performed a short time-course experiment. Samples containing 45 copepods per replicate were electroporated in assay mixture with target dsRNA ($n = 3$) or no dsRNA (untreated control, $n = 5$). Samples were then cultured as before, and relative expression of target genes examined by qPCR on days 2, 5 and 8 post-treatment. *T*-tests were then used to assess whether target gene expression was significantly suppressed at each sampled day.

Quantitative PCR (qPCR)

Transcription levels of all experiments above were monitored by qPCR. From each sample, cDNA was synthesized from 150 ng of total RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems), and the cDNA was then diluted to 3 ng/μL (RNA equivalent). qPCRs were performed in a total of 15 μL containing 3 μL (9 ng) of template, 1× iTaq Universal SYBR Green supermix (Bio-Rad), and 0.35 μM of each primer, and were run on a Stratagene MX3000P (Agilent) system with the following cycling parameters: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 58 °C for 30 s. After each run, a dissociation (melting) curve step was carried out to check for primer–dimers or nonspecific amplifications. Relative expression of each target gene in each sample was quantified with the $2^{-\Delta C_t}$ method (Schmittgen & Livak 2008), using the geometric average of the expression levels of *myosin* and *GAPDH* genes for normalization. These two genes were previously shown to be suitable reference genes for qPCR in *T. californicus* (Schoville *et al.* 2012), and we performed additional tests to show their stability in our electroporated samples (Appendix S1, Supporting information). All qPCRs were performed in duplicate or triplicate.

Before using new qPCR primers, their amplification efficiencies were estimated following Pfaffl (2001). cDNAs equivalent to 48 pg, 240 pg, 1.2 ng, 6 ng and 30 ng of total RNA were used as templates in triplicate qPCRs for each gene. Efficiencies were calculated as $E = 10^{[-1/m]}$, where m is the slope of the regression of C_t values by $\log_{10}$(ng of template). Only primer pairs with $E \geq 1.90$ were considered appropriate for use. Primer information can be found in Table 1.

Results

RNAi-mediated knock-down of hspb1

Both feeding and rehydration methods are simple, use relatively few reagents/materials and result in the lowest experimental mortality of copepods (Table 2). However, qPCR detected no evidence of *hspb1* knock-down in any replicate within these approaches. The use of lipofection reagent resulted in a few instances of gene knockdown, but suppression levels were inconsistent and weak and accompanied by high copepod mortality. Similarly, high experimental mortality and low and inconsistent *hspb1* knock-down were observed in electroporation trials with high voltages (>125 V). Once electroporation voltage was lowered to 50 V, with multiple pulses of moderate length (10–20 ms), *hspb1* knock-down was consistently >50% [% knock-down = (1 − expression relative to control) × 100], despite a few replicates still showing low RNAi. This pattern was slightly improved by applying a short higher voltage pulse (100 V–5 ms) before the multiple 50 V pulses (Table 2). This improvement may be caused by better tissue permeabilization at high voltage followed by electrophoretic movement of dsRNA during longer pulses of low voltage (Bureau *et al*. 2000).

After achieving knock-down of *hspb1* consistently above 50% with delivery by electroporation, we examined suppression of this gene every 2 days during an 8-day period. During the time-course experiment, relative *hspb1* expression varied significantly among treatment groups (ANOVA: $F_{2,48} = 81.72$, $P < 3 \times 10^{-15}$). Specifically, relative *hspb1* expression in samples treated with *hspb1* dsRNA was significantly reduced (range: 0.087–0.514, or conversely 49.6–91.3% knockdown) compared to that of both control groups at all four time points sampled (Tukey's tests, all $P < 0.003$; Fig. 1). In turn, delivery of control dsRNA did not result in reduced *hspb1* expression (Tukey's tests for comparisons with untreated control, all $P > 0.97$). In the *hspb1* dsRNA group, knock-down of *hspb1* varied significantly among days (ANOVA: $F_{3,16} = 3.29$, $P = 0.0479$), but this was largely due to a small yet significant increase in expression (i.e. decrease in knock-down level) from day 2 to day 8 (Tukey's test, $P = 0.0307$). Overall, the strongest RNAi effect was observed on day 4, when most replicates exhibited >80% knock-down of *hspb1* (Fig. 1). Finally, we verified that *hspb1* dsRNA did not trigger transcriptional suppression of *hsp70* or *serpin* (both $P > 0.80$; Fig. 1).

Hspb1 expression and tolerance to high heat stress

To examine the phenotypic effect of *hspb1* suppression in *Tigriopus californicus*, we electroporated pools of copepods in assay mixtures containing *hspb1* dsRNA ($n = 10$), control dsRNA ($n = 6$) or no dsRNA ($n = 6$). In the target group, knock-down ranged from 49.5% to 87.5%, showing consistent *hspb1* suppression across replicates (overall ANOVA: $F_{2,19} = 49.4$, $P < 10^{-7}$; Tukey's test, $P < 0.0001$ in comparisons to control groups; Fig. 2a). Control

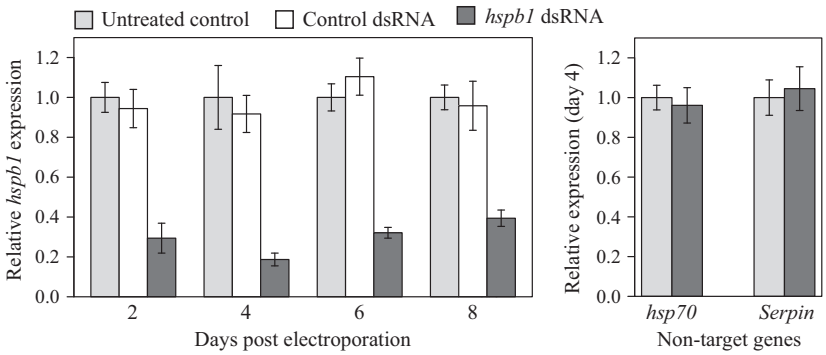

Fig. 1 Time-course of heat-shock beta 1 (*hspb1*) knock-down via RNAi in *Tigriopus californicus*. Copepods were electroporated in assay mixture containing *hspb1* dsRNA ($n = 5$), nonspecific control dsRNA ($n = 5$) or no dsRNA ($n = 5$), and gene expression was quantified every 2 days postelectroporation. The right panel shows relative expression of two nontarget genes. Shown are mean ± SE expression (relative to *myosin* and *GAPDH* expression). Relative expression values were scaled to the mean of untreated controls at each day. Compared to both control groups, *hspb1* dsRNA significantly suppressed *hspb1* expression at all sampled time points ($P < 0.003$) but did not affect expression of nontarget genes ($P > 0.80$).

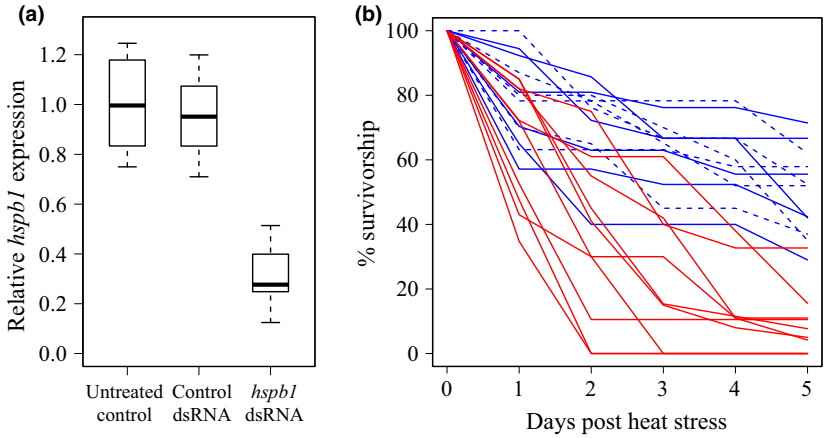

Fig. 2 Tolerance of *Tigriopus californicus* to high thermal stress during heat-shock beta 1 (*hspb1*) suppression. Copepods were electroporated in assay mixture containing *hspb1* dsRNA (n = 10, red lines), nonspecific control dsRNA (n = 6, dashed blue lines) or no dsRNA (n = 6, solid blue lines). Four days after treatment, subsamples of copepods were used to quantify (a) expression of *hsbp1* (relative to *myosin* and *GAPDH*). The remaining subsamples were exposed to a high temperature stress (36 °C for 1 h), and (b) their survivorship was monitored for 5 days. This experiment was performed separately after determining the time-course of *hspb1* (Fig. 1).

dsRNA again did not trigger *hspb1* RNAi (Tukey's test, P = 0.863; Fig. 2a).

Mortality due to heat stress occurred in all treatment groups, but samples treated with *hspb1* dsRNA showed higher mortality than controls already at day 1 post heat exposure (Fig. 2b). Kaplan–Meier curve analyses showed a highly significant overall effect of treatment on survivorship of copepods during the first 5 days post heat stress (χ^2 = 145, d.f. = 2, P < 0.0001). Comparisons of curves between treatment groups revealed that copepods electroporated with control (nonspecific) dsRNA showed no difference compared to untreated controls (χ^2 = 0.054, d.f. = 1, P = 0.816). However, samples treated with *hspb1* dsRNA suffered dramatically reduced survival after heat stress compared to controls (mean ± SE % survivorship at day 5, *hspb1* dsRNA: 8.8 ± 3.2, control dsRNA: 48.9 ± 4.5, untreated control: 51.1 ± 7.0; χ^2 > 93, both P < 0.0001). Three of the 10 *hspb1* dsRNA replicates had no survivors by day 5 post heat stress (Fig. 2b).

RNAi of additional genes

We tested whether our working method of dsRNA delivery could be used for suppression of other target genes without further optimization. In all four genes examined, treatment with target dsRNA triggered significant knock-down of target genes on at least day 2 postelectroporation (Fig. 3a,b,d,e; *t*-tests of comparison to controls, *hsp70*: P = 0.0058; *serpin*: P = 0.019; *mtRPOL*: P = 0.0019; *mRPS29*: P = 0.0074); expression of a nontarget gene (*hspb1*) was not affected (Fig. 3c,f). *Hsp70*, *serpin* and *mtRPOL* continued to be significantly suppressed by day 5 (*t*-tests, *hsp70*: P = 0.031; *serpin*: P = 0.029; *mtRPOL*: P = 0.028), while expression of *mRPS29* was no longer significantly reduced compared to its control (P = 0.59). By day 8, *hsp70* was still consistently below control levels (*t*-test, P < 0.001), but expression levels of *serpin* and *mtRPOL* returned to control levels (*serpin*: P = 0.25; *mtRPOL*: P = 0.23).

Discussion

Transcriptome sequencing studies have revolutionized studies in both model and nonmodel organisms by efficiently highlighting genes putatively involved in developmental, physiological and genetic processes of interest. The copepod *Tigriopus californicus* has served as an excellent system for studies of allopatric speciation, hybridization, physiology and adaptation (Burton *et al.* 2006). Schoville *et al.* (2012) used RNA-seq to show that when exposed to high temperatures, *T. californicus* from San Diego upregulate certain *hsps* to over 100-fold their constitutive levels. To determine the relative importance of these genes, the phenotypic effect of individual genes should be isolated. In this study, we demonstrate an RNA interference protocol that can be used to test functional hypotheses in *T. californicus*.

Because of their small size, copepods were electroporated in pools of 15 or more individuals to consistently obtain high-quality RNA. Quantitative PCR showed that samples treated with *hspb1* dsRNA repeatedly suffered approximately 50–90% reduction in *hspb1* transcript levels when compared to animals exposed to a negative control dsRNA. This comparison confirms that suppression of *hspb1* was not caused by a global immune response during exposure to foreign dsRNA, which is known to

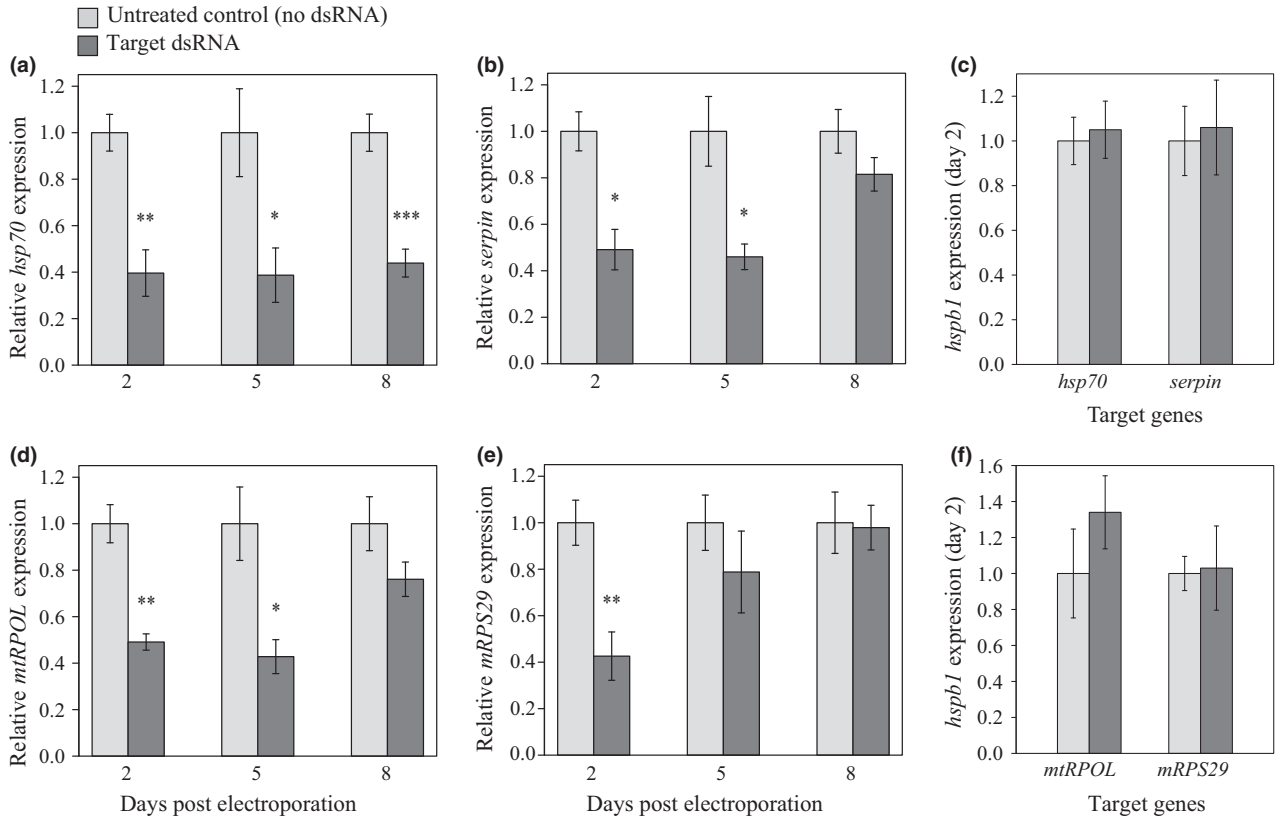

Fig. 3 Relative expression (mean ± SE) of four target genes following electroporation with respective dsRNA. Genes tested were (a) heat-shock protein 70, (b) serine protease inhibitor, (d) mitochondrial RNA polymerase and (e) mitochondrial ribosomal protein S29. Copepods were electroporated in assay mixture containing respective target dsRNA (n = 3) or no dsRNA (n = 5), and gene expression was quantified two, five and 8 days post-electroporation. The expression of a nontarget gene (*hspb1*) was also quantified (c and f). T-tests: *P < 0.05, **P < 0.01, ***P < 0.001.

occur in mammalian systems (Elbashir *et al.* 2001). Knock-down of *hspb1* was detectable as early as 2 days after treatment with target dsRNA, and it lasted for at least 8 days. Such stable gene suppression allowed us to directly test the role of *hspb1* in thermotolerance of copepods from San Diego.

Knock-down of *hspb1* resulted in clear and dramatic decrease in tolerance to acute high temperature stress. Copepods treated with control dsRNA or with no dsRNA suffered approximately 50% mortality within 5 days after exposure to 36 °C. This was the expected 'background' level of mortality based on previous experiments (Willett 2010; Kelly *et al.* 2012). When copepods were treated with *hspb1* dsRNA, mortality accumulated rapidly, surpassing that of control levels. This occurred even in replicates for which knock-down was relatively less potent (<70%). Therefore, even incomplete suppression of *hspb1* was sufficient to increase copepod mortality, indicating that strong upregulation of this gene during acute stress is essential for thermotolerance in this population. Our original hypothesis for the importance of *hspb1* was based on its high induction during experimental heat stress (>100-fold upregulation, Schoville *et al.* 2012). Alternatively, *hspb1* may be centrally located in a gene network (Zhang *et al.* 2012; Fu *et al.* 2014), such that a significant suppression of its expression generates detrimental cascading effects on multiple genes. Future experiments integrating RNA-seq and RNAi within a systems biology framework (Iancu *et al.* 2012) will provide a powerful means of understanding complex functional pathways.

While the current RNAi protocol was developed and optimized with one gene, our goal was to establish a method that can be more generally applied to other genes. We tested our approach with four additional genes of interest and obtained promising results. Expression of all genes was knocked down by 50% or higher in at least one of the assayed periods. Suppression of *hsp70* was the most potent and stable; relative *hsp70* expression remained below 0.50 for at least 8 days, and it reached as low as 0.21 in some replicates. While these knock-down levels were not as strong as those achieved with *hspb1*, they are well within those commonly obtained in vivo in a wide range of model systems (Zhou *et al.* 2008; Stefanic

et al. 2010; Tran *et al.* 2010; Chen *et al.* 2012; Benoit *et al.* 2014). Moreover, efficiency of RNAi in an animal model is highly variable among genes, and each gene may require slightly different treatment conditions for potent and stable knock-down (Bellés 2010; Stefanic *et al.* 2010; Terenius *et al.* 2011). Our results, which were obtained without further optimization, are hence highly encouraging.

Because of such variation, a few key factors must be considered and tested when designing RNAi experiments targeting a new gene. Our trials employed one 'version' of dsRNA for each target gene. However, the length of dsRNA as well as the position within a targeted mRNA sequence is known to significantly influence RNAi efficiency. For instance, Sukno *et al.* (2007) and Chen *et al.* (2012) found, in the nematodes *Heterodera glycines* and *Trichinella spiralis*, respectively, that a dsRNA designed from a 3′ region of the target genes triggered more potent suppression than similarly sized dsRNAs targeting 5′ regions. In contrast, in a different nematode (*Nippostrongylus brasiliensis*), a 240-bp dsRNA designed from the 5′ region of the target gene resulted in more potent knock-down than a 1799-bp dsRNA derived from the full coding sequence (Hussein *et al.* 2002). In the latter study, the smaller dsRNA also resulted in more stable suppression (lasting > 6 days) compared to the larger dsRNA design (<4 days). Therefore, functional studies employing RNAi in *T. californicus* should include preliminary trials using multiple dsRNA designs to obtain optimal knock-down of target genes.

In three of the five genes examined in our study, expression returned to control levels in <8 days post-treatment. As mentioned above, different dsRNA designs may improve RNAi stability. However, the intrinsic dynamics of the target gene's expression may also influence RNAi sensitivity. Persistence of RNAi effects can be correlated with endogenous rates of mRNA turnover (Gazzani *et al.* 2004; Lilley *et al.* 2012), while genes with efficient feedback mechanisms may increase transcription rates as a means of counteracting mRNA depletion (Bellés 2010). Time-course trials such as the ones performed here are essential for determining the longevity of gene knock-down. Even if the RNAi effect is shown to be short-lived, knowledge of knock-down time-course will serve as guide for choosing when phenotypes can be examined (Shakesby *et al.* 2009) or when re-treatment with dsRNA can extend RNAi longevity (Ambrosone *et al.* 2012; Rouhana *et al.* 2013).

In conclusion, we report a relatively low-cost and high-throughput RNAi method for target gene knock-down in the emerging model *T. californicus*. Electroporation of copepods with dsRNA designed from mRNA sequences of five candidate genes resulted in repeated reductions of their expression levels. While optimization of treatment conditions and reagents is necessary for optimal gene knock-down, our protocol serves as an excellent starting point for functional genetic studies in this species.

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F.S.B., S.D.S. and R.S.B. designed the study. F.S.B. and S.D.S. performed the laboratory work. F.S.B. analysed the data and wrote a first version of the manuscript. S.D.S. and R.S.B. helped write the manuscript.

Data accessibility

Raw C_t values for the three main experiments (Exp 1: *hspb1* time-course; Exp 2: *hspb1* knock-down and thermotolerance; and Exp 3: knock-down of four additional genes) as well as survivorship data from Experiment 2 were deposited in the Dryad Digital Repository (doi:10.5061/dryad.5cb17).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Assessment of expression stability of *GAPDH* and *myosin* for qPCR in *Tigriopus californicus*.

Fig. S1 Quantification of *GAPDH*, *myosin*, and *hspb1* expression in control and electroporated copepods.