

Sensitivity to near-future CO₂ conditions in marine crabs depends on their compensatory capacities for salinity change

Nia M. Whiteley, Coleen C. Suckling, Benjamin J. Ciotti, James Brown, Ian D. McCarthy, Luis Gimenez and Chris Hauton

Table S1. Measured and estimated seawater treatment parameters for both species.

Species	Parameter	SW + CCO ₂ (control)	SW + HCO ₂	DW + CCO ₂	DW + HCO ₂
<i>Carcinus</i>	pH_{NIST}	8.03 ± 0.01^a	7.72 ± 0.01^b	8.09 ± 0.01^c	7.78 ± 0.01^d
<i>maenas</i>	Salinity	33.22 ± 0.04^a	33.17 ± 0.05^a	25.47 ± 0.09^b	25.39 ± 0.07^b
	Temp (°C)	12.53 ± 0.06^a	12.71 ± 0.06^a	12.58 ± 0.05^a	12.64 ± 0.06^a
	A_T (μmol kg⁻¹)	2,272 ± 4^a	2,272 ± 4^a	1,813 ± 5^b	1,825 ± 5^b
	DIC (μmol kg⁻¹)	2,120 ± 4^a	2,222 ± 5^b	1,716 ± 7^c	1,795 ± 4^d
	<i>p</i> CO ₂ (μatm)	527 ± 13 ^a	1125 ± 56 ^b	508 ± 49 ^a	930 ± 27 ^b
	Ω calcite	2.80 ± 0.06 ^a	1.54 ± 0.05 ^b	1.88 ± 0.07 ^b	1.06 ± 0.03 ^c
	Ω aragonite	1.78 ± 0.04 ^a	0.98 ± 0.03 ^b	1.16 ± 0.05 ^b	0.66 ± 0.02 ^c
<i>Cancer</i>	pH_{NIST}	7.99 ± 0.01^a	7.67 ± 0.01^b	8.03 ± 0.001^c	7.77 ± 0.01^d
<i>pagurus</i>	Salinity	33.17 ± 0.05^a	33.27 ± 0.05^a	25.60 ± 0.09^b	25.32 ± 0.08^b
	Temp (°C)	12.66 ± 0.09^a	12.78 ± 0.09^a	12.75 ± 0.08^a	12.68 ± 0.09^a
	A_T (μmol kg⁻¹)	2,303 ± 16^a	2,297 ± 10^a	1,821 ± 13^b	1,826 ± 6^b
	DIC (μmol kg⁻¹)	2,143 ± 8^a	2,243 ± 10^b	1,721 ± 13^c	1,787 ± 9^d
	<i>p</i> CO ₂ (μatm)	570 ± 45 ^a	1138 ± 77 ^b	481 ± 30 ^a	874 ± 56 ^b
	Ω calcite	3.01 ± 0.28 ^a	1.61 ± 0.08 ^b	1.91 ± 0.08 ^b	1.17 ± 0.05 ^c
	Ω aragonite	1.92 ± 0.18 ^a	1.03 ± 0.05 ^b	1.19 ± 0.05 ^b	0.73 ± 0.03 ^c

A_T = total alkalinity, DIC = dissolved inorganic carbon, SW = full strength seawater, DW = diluted seawater, CCO₂ = ambient *p*CO₂, HCO₂ = elevated *p*CO₂.

Values are means ± SEM.

Data represent daily values for pH, salinity and temperature and monthly values for remaining parameters.

Measured values shown in bold used to calculate *p*CO₂, calcite and aragonite saturation states (Ω calcite and Ω aragonite).

Different letters indicate significant differences among treatments (*P* < 0.05) using one-way ANOVA or Kruskal-Wallis test, and appropriate *post hoc* tests.

Table S2. Details of gene targets, primers and assay performance for real-time PCR analyses.

Target	Genbank accession no.	F primer	R primer	Amplicon size (bases)	F / R primer conc. (μM)	Efficiency	Linear range (μg in 50μL)
<i>Carcinus maenas</i>							
<i>act</i>	MG554415	TCACCAACTGGGAC GACATG	GCCACACGGAGCTCAT TGTAG	64	0.3 / 0.3	1.009	0.4 - 0.0002
<i>AK</i>	AF167313.1	CCAATGCTTGCCGCT ACTG	AGGAAGGTCTTGTGT CGTTGTG	64	0.3 / 0.3	0.976	0.4 - 0.0002
<i>AE</i>	CX994129	CCTGCTTGTGTGCG AACAGT	GATTACACAGGTGCAT GTGTAGA	74	0.3 / 0.3	0.995	0.2 - 0.0002
<i>CAC</i>	EU273943.1	CATAAAGGGCAGGC GATCAG	ACCGTTCTTGGGTAGG AAGGTT	66	0.3 / 0.3	1.040	0.2 - 0.002
<i>gpi-CA</i>	EU273944.1	CACGCCTACCTGCA ACGAA	TGACGCTCGGAGATG GAGAT	69	0.3 / 0.3	1.059	0.2 - 0.0002
<i>eef1A</i>	MG430409	TCTGGATGGAGGCT CAATGTT	AGAGCGGCAGCTATG AGTTCA	65	0.3 / 0.3	0.924	0.4 - 0.00002
<i>gapdh</i>	MG430408	TGCGGTGTGAATTT GGAGAA	GGTGCAGGAGGCATT GGAT	63	0.3 / 0.3	1.007	0.4 - 0.0002
<i>NKAα</i>	AY035550.1	GAGGGATGGAAGG CTCTCTCTAG	ACGTCATCTTGGGCAG TCTTG	74	0.3 / 0.3	1.087	0.4 - 0.002
<i>NHE</i>	U09274.1	GCGCCTCCACCTACA AGAGA	TGGCCGATATCCGATT GTG	67	0.3 / 0.3	1.129	0.2 - 0.0002
<i>tub</i>	MG430410	CTCTGCTTCTTTGCG GATAACA	CACTACACGGAAGGT GCTGAAC	63	0.3 / 0.3	0.972	0.4 - 0.0002
<i>VATB</i>	AF189779.2	CATGATGACCACAA GGATAACTTTG	CCTGGCAGTCTCCATG TTGA	69	0.3 / 0.3	0.973	0.4 - 0.002
<i>Cancer pagurus</i>							
<i>act</i>	FR687021.1	GTGACATCAAGGAA AAGCTGTGTT	GGAAGCGGCAGTGGT CAT	68	0.05 / 0.05	0.933	0.4 - 0.000014
<i>AK</i>	MF972215	GATGACCACTTCCTC TTC	GTCGTTGTTGTGGTAG AT	99	0.3 / 0.3	0.966	0.4 - 0.000014
<i>AE</i>	MF972216	CTGAGCACAAAGGGA AATACTCATTT	CCTCTCCACCATAT GTTGGA	78	0.3 / 0.05	1.012	0.4 - 0.0014
<i>CAC</i>	MF972217	ATGGCGAGACCCCT GTGACT	GAACACCCGGAGATG AAAAAGA	65	0.3 / 0.3	0.905	0.4 - 0.0014
<i>gpi-CA</i>	MF972218	TTTGGCAGTACTTG GTGTGATGT	AGGAGCAAGAGCCGG GTTA	61	0.3 / 0.3	0.972	0.4 - 0.00004
<i>eef1A</i>	MF972219	AACCACCCTGGTCA GATCCA	TGCAGGCAATATGTGC AGTGT	70	0.3 / 0.3	0.910	0.4 - 0.00014
<i>gapdh</i>	MF972220	TCCCCGAGTTGAAT GGAAAG	AGATCCACCACGGAC ACATCA	73	0.05 / 0.05	0.984	0.4 - 0.00014
<i>NKAα</i>	MF972221	CCAGAGACTCAACA TTCC	GTCATCCAGTTGTTCA GA	106	0.3 / 0.3	1.005	0.4 - 0.00014

<i>tub</i>	MF972222	CCCTACCCAGAATC CACTTC	CATGGTAAGCCTTCTC AGCAGAA	70	0.05 / 0.05	0.968	0.4 - 0.000004
<i>VATB</i>	MF972223	CTGACTATGCCTAAT GATGA	TAGATTTGACGGTTGT GAA	107	0.3 / 0.3	0.982	0.4 - 0.0014

Abbreviations: actin, *act*; arginine kinase, *AK*; anion exchange protein, *AE*; cytoplasmic carbonic anhydrase, *CAC*; glycosyl-phosphatidylinositol-linked carbonic anhydrase, *gpi-CA*; elongation factor 1 A, *eef1A*; glyceraldehyde-3-phosphate dehydrogenase, *gapdh*; Na⁺/K⁺ ATPase alpha subunit, *NKAα*; Na⁺/H⁺ exchanger, *NHE*; tubulin, *tub*; and vacuolar-type H⁺ ATPase subunit B, *VATB*.

New Accession numbers in bold represent targets sequenced for this study.

Linear range refers to the concentration of the original mRNA sample in the final 50 µL real-time PCR reaction mix.

Table S3. Model selection for the physiological variables in *Carcinus maenas*.

Full term	Term removed	pH AICc	pCO ₂ AICc	[HCO ₃ ⁻] AICc	Osmolality AICc	NKA AICc
4-way factorial		-189.77	324.2	602.1	1237.4	298.0
3-way factorial		-208.65	312.9	590.9	1228.5	288.8
3-way terms with CW		-200.97	302.2	580.1	1221.9	282.1
	CW:pCO ₂ :Time	-218.06	292.7	570.1	1213.1	277.5
	CW:Sal:Time	-217.71	293.5	572.6	1216.7	274.4
	CW:Sal:pCO ₂	-209.37	299.4	578.0	1218.4	278.4
2-way factorial		-224.29	283.1	563.2	1209.7	266.3
	pCO ₂ :Time	-231.47	274.7	554.3	1201.2	258.0
	Sal:pCO ₂	-224.47	280.7	562.0	1206.9	263.2
	Sal:Time	-232.55	275.2	556.1	1207.9	263.2
2-way with CW		-240.13	265.3	546.2	1197.5	253.5
	CW:Time	-247.10	260.0	541.9	1192.1	248.6
	CW:Sal	-242.45	263.0	543.7	1197.3	250.9
	CW:pCO ₂	-241.95	266.4	545.7	1194.9	250.9
Additive		-250.80	258.0	538.5	1189.8	243.9
	Sal	-250.00	256.2	536.2	1221.8	298.8
	pCO ₂	-252.40	256.0	536.2	1187.4	244.7
	Time	-248.60	261.6	546.5	1196.8	241.5
	CW	-252.90	256.0	536.7	1188.1	243.8

Abbreviations: CW = carapace width; Sal = salinity; pCO₂ = partial pressure of CO₂; NKA = Na⁺/K⁺-ATPase activity.

Models selected using generalised linear square models and Akaike Information Criteria (AIC_c) for all dependent variables (haemolymph acid-base status and osmolality, and gill NKA activities). Terms in bold are retained in the models.

Table S4. Likelihood ratio tests (LR tests) for the selected models in *C. maenas*.

Variable	Term	AICc	LR test	P
Haemolymph pH	Time	-248.60	9.09	0.028
Haemolymph pCO ₂	Time	261.6	10.50	0.015
Haemolymph [HCO ₃ ⁻]	Time	546.5	14.92	0.002
Haemolymph osmolality	Salinity	1221.8	13.96	0.003
	Time	1196.8	34.41	<0.001
Branchial NKA activity	Salinity	298.8	57.28	<0.001

P values correspond to comparison of full term (S3) and a reduced model lacking the respective term.

Table S5. Model selection for physiological variables in *Cancer pagurus*.

Full term	Term removed	pH AICc	pCO ₂ AICc	[HCO ₃ ⁻] AICc	Osmolality AICc	NKA AICc
4-way factorial:		-106.0	448.8	632	1386.5	107.1
3-way factorial		-116.3	437.2	622	1386.5	102.8
3-way terms with CW		-126.6	433.7	616	1382.0	95.6
	CW:pCO ₂ :Time	-133.7	425.6	607	1375.5	96.9
	CW:Sal:Time	-131.4	424.6	607	1375.8	86.1
	CW:Sal:pCO ₂	-130.1	431.5	615	1378.6	92.8
2-way factorial		-141.7	415.7	599	1366.6	85.1
	pCO ₂ :Time	-148.7	408.2	591	1361.2	77.8
	Sal:pCO ₂	-144.7	413.2	596	1365.7	82.2
	Sal:Time	-149.2	411.5	595	1361.0	77.2
2-way with CW		-158.3	402.8	586	1355.4	68.0
	CW:Time	-165.3	396.0	579	1357.7	65.5
	CW:Sal	-160.6	400.2	584	1352.9	65.5
	CW:pCO ₂	-160.3	401.7	584	1357.8	67.5
	Sal				1511.0	
Additive		169.4	392.5	577	1358.3	62.5
	Sal	-164.0	390.3	575	1503.3	60.2
	pCO ₂	-170.5	396.3	583	<i>1361.0</i>	60.7
	Time	-143.5	420.4	599	<i>1361.4</i>	59.6
	CW	-170.3	403.8	582	-	67.5

Abbreviations: CW = carapace width; Sal = salinity; pCO₂ = partial pressure of CO₂; NKA = Na⁺/K⁺-ATPase.

Models selected using generalised linear square models and Akaike Information Criteria (AIC_c) for all dependent variables (haemolymph acid-base status and osmolality, and gill NKA activities). Terms in bold are retained in the models.

Table S6. Likelihood ratio tests (LR test) for the selected models in *C. pagurus*.

Variable	Term	AICc	LR test	P
Haemolymph pH	Salinity	-164.0	7.90	0.005
	Time	-143.5	33.07	<0.001
Haemolymph pCO ₂	pCO ₂	396.3	6.23	0.013
	Time	420.4	35.06	<0.001
	CW	403.8	13.78	<0.001
Haemolymph [HCO ₃ ⁻]	pCO ₂	583.0	9.06	0.003
	Time	599.0	28.98	<0.001
	CW	582.0	7.64	0.006
Haemolymph osmolality	CW:pCO ₂	1357.8	4.90	0.027
	CW:Time	1357.7	9.69	0.021
	Salinity	1511.0	160.50	<0.001
Branchial NKA activity	CW	67.5	7.371	0.007

P values correspond to comparison of full term (S5) and a reduced model lacking the respective term.

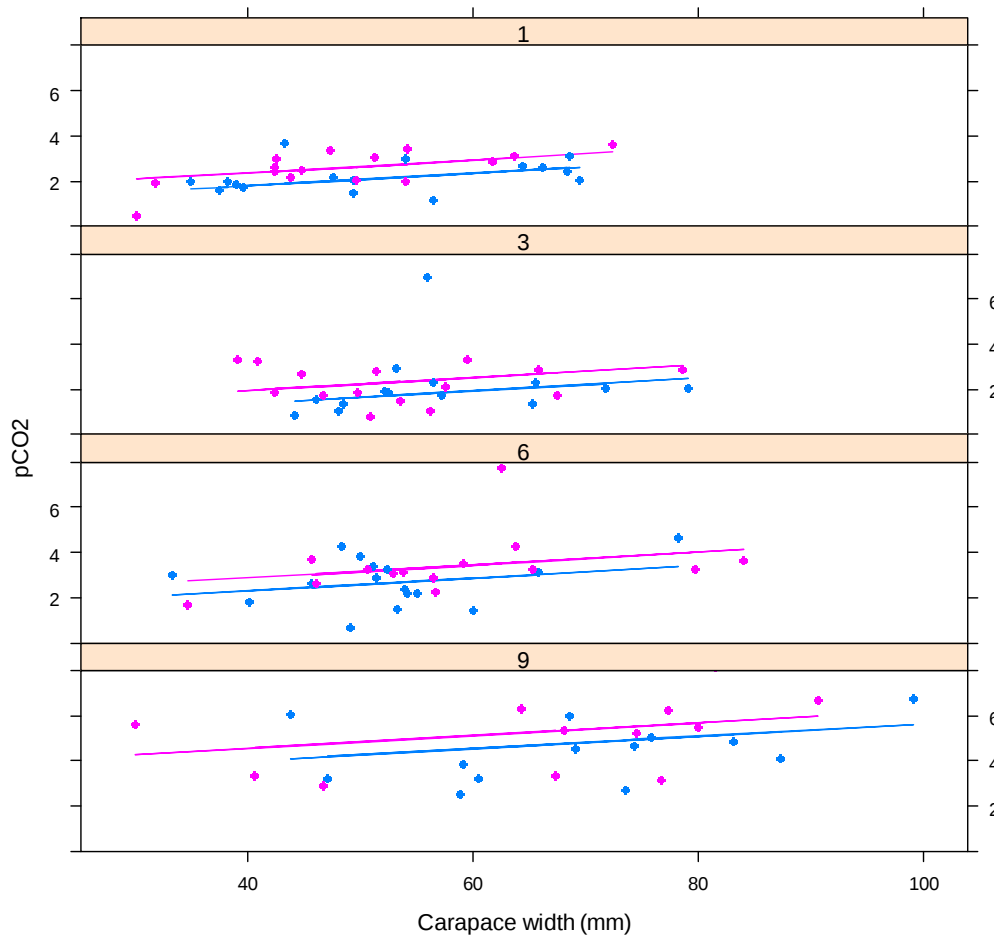


Figure S1. Relationship between body size expressed as carapace width and haemolymph $p\text{CO}_2$ (kPa) for individual *Cancer pagurus* exposed to control $p\text{CO}_2$ (blue lines and symbols) and elevated $p\text{CO}_2$ (pink line and symbols) at different sampling times (1, 3, 6 and 9 months). Lines represent predicted values, and individual data points are observed values. The model parameters suggest a consistent effect of CO_2 levels irrespective of the effect of time and body size.

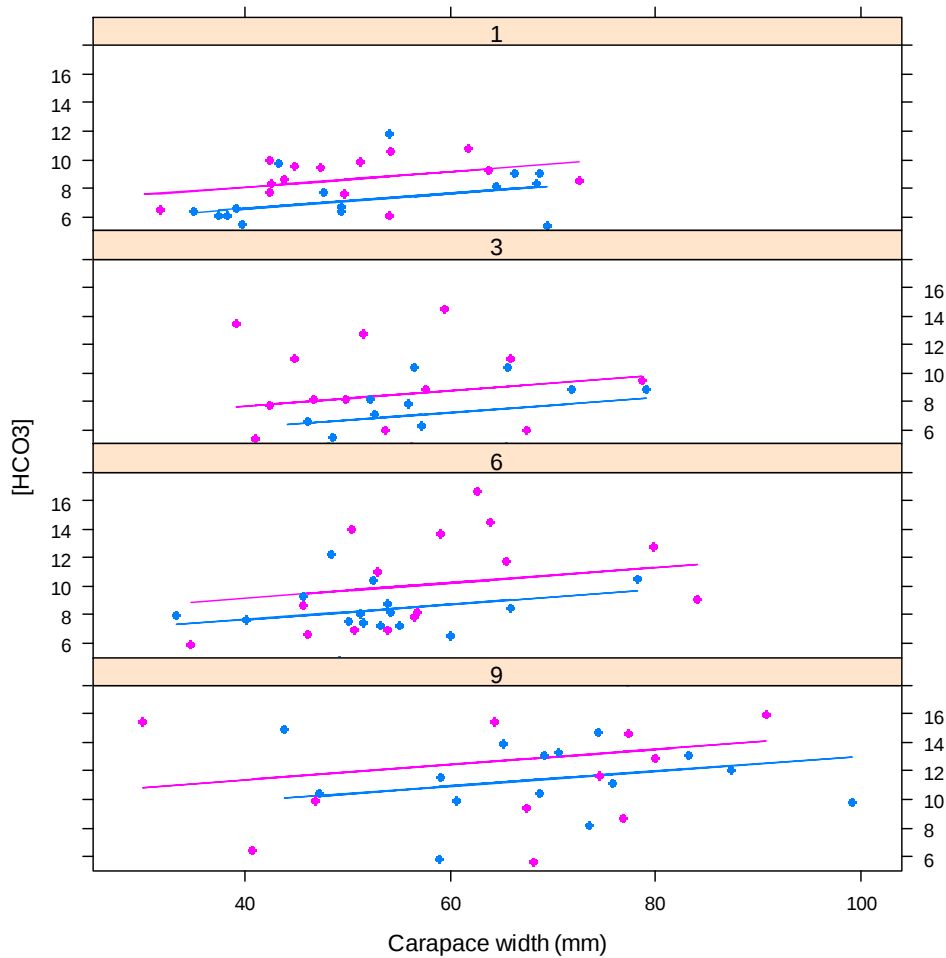


Figure S2. Relationship between body size expressed as carapace width and haemolymph $[\text{HCO}_3^-]$ (mmol l^{-1}) for individual *Cancer pagurus* exposed to control $p\text{CO}_2$ (blue lines and symbols) and elevated $p\text{CO}_2$ (pink line and symbols) at different sampling times (1, 3, 6 and 9 months). Lines represent predicted values, and individual data points are observed values. The model parameters suggest a consistent effect of CO_2 levels irrespective of the effect of time and body size.

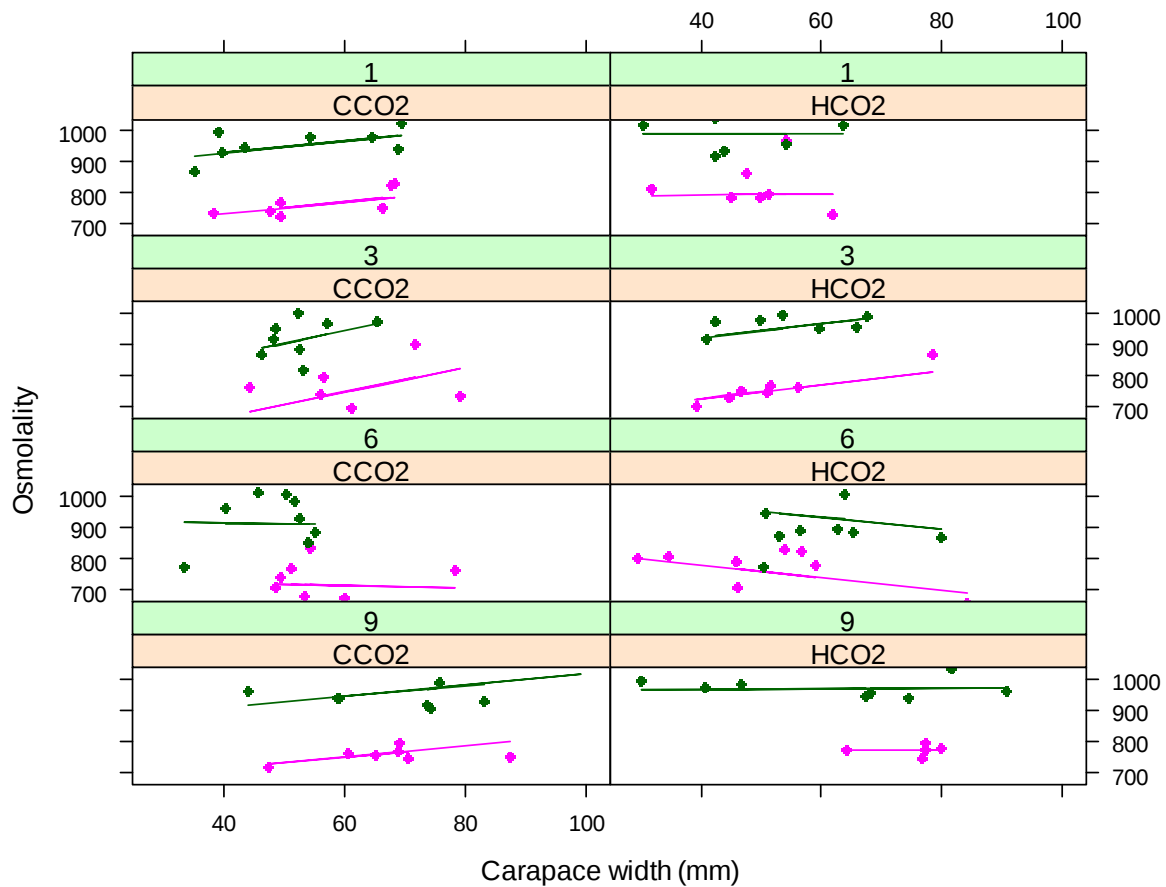


Figure S3. Observed and predicted effects of body size (carapace width), CO₂ (C= control pCO₂, H= elevated pCO₂), sampling times (1, 3, 6 and 9 months) and salinity (green line and symbols = full strength SW; pink lines and symbols = DW) on haemolymph osmolality (mosmol kg⁻¹) in *Cancer pagurus*. Predictions are based on parameter estimates.

Table S7. AICc values and outcomes from model selection for gene expression (log(CNRQ)) in *Carcinus maenas*.

Full model	Term removed	<i>AE</i> AICc	<i>CAC</i> AICc	<i>gpi-CA</i> AICc	<i>NKAα</i> AICc	<i>NHE</i> AICc	<i>VATB</i> AICc
4-way factorial		87.90	240.9	191.3	120.24	159.6	102.19
	CW:Sal:pCO ₂ :Time	80.00	246.2 ^c	186.7	110.32	151.1	95.05
3-way factorial		80.00	246.2	186.7	110.32	151.1	95.05
	Sal:pCO ₂ :Time	78.33	243.0	180.0	101.05	150.3	85.85
3-way terms with CW		78.33	243.0	180.0	101.05	150.3	85.85
	CW:pCO ₂ :Time	69.58	248.6	170.8	96.54	150.9 ^{b,c}	78.46
	CW:Sal:Time	70.99	245.9	171.1	99.64	146.7	78.35
	CW:Sal:pCO ₂	75.95	240.5	178.3	102.22 ^{b,c}	147.3	84.42
2-way factorial		62.13	<i>245.1</i>	162.8	95.68	145.8	71.61
	pCO ₂ :Time	55.54	<i>238.6</i>	156.0	89.14	139.7	63.87
	Sal:pCO ₂	62.76 ^a	<i>243.0</i>	160.5	96.07 ^a	145.0	69.44
	Sal:Time	55.00	<i>237.4</i>	157.4	89.94	140.0	70.29
2-way with CW		50.41	<i>229.5</i>	149.2	84.16	134.4	61.46
	CW:Time	56.21	<i>224.2</i>	148.4	79.25	142.4	58.62
	CW:Sal	48.03	<i>227.6</i>	146.7	81.77	137.3	59.02
	CW:pCO ₂	47.86	<i>227.1</i>	147.6	86.64 ^d	134.4	61.62 ^a
Additive		52.68	<i>219.9</i>	146.0	77.70	143.4	55.87
	Sal	51.44	<i>264.0</i>	151.0	94.95	<i>146.2</i>	62.66
	pCO ₂	50.94	<i>217.6</i>	143.8	78.14 ^a	142.9	53.72
	Time	<i>51.21</i>	<i>221.2</i>	140.5	74.87	<i>140.1</i>	52.80
	CW	<i>53.72</i>	<i>220.2</i>	144.6	78.27 ^a	<i>155.3</i>	53.63

Further investigation of the higher order interactions for *CAC*, *NKAα* and *NHE* were conducted to test for biological relevance via individual plots of all factor combinations, and by simplifying the models according to AICc criteria and LR tests.

a Removed because log-likelihood ratio test not significant (alpha = 0.05)

b Log-likelihood ratio test significant (alpha = 0.05)

c interaction removed to explore simpler models

d Could be removed from the best model (AICc for Additive + CW: pCO₂ = 76.94; AICc for Additive = 77.70, LLR test not significant (alpha = 0.05)).

Bold text: terms retained in the final model.

Grey text & italics: terms that cannot be tested due to higher order interactions

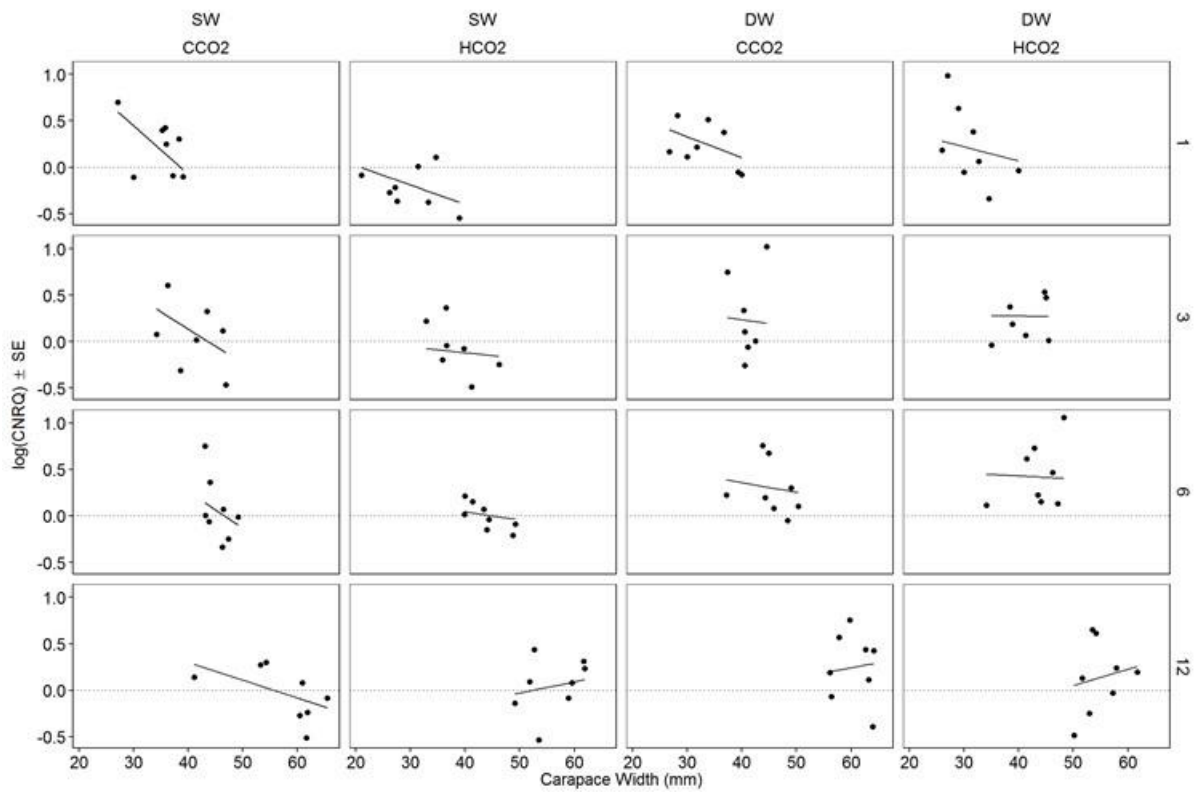


Figure S4. Relationship between $NKA\alpha$ expression in the posterior gills of *Carcinus maenas* and body size given as carapace width in all 4 factor combinations: SW/control $p\text{CO}_2$; SW/ elevated $p\text{CO}_2$; DW/control $p\text{CO}_2$; DW/elevated $p\text{CO}_2$, where SW = seawater at a salinity of 33 and DW = dilute seawater at a salinity of 25. Gene expression shown as differences in $\log(\text{CNRQ})$. Straight lines constructed from parameter estimates. Horizontal broken line represents the mean for baseline crabs. Overall, figure demonstrates that although the CW:Sal; $p\text{CO}_2$ term was significant (Table S7), the relationship between $NKA\alpha$ expression and carapace width is inconsistent among salinity and $p\text{CO}_2$ treatments: simpler models were therefore explored. SW = seawater at a salinity of 33; DW = dilute seawater at a salinity of 25; CCO₂ = ambient $p\text{CO}_2$; HCO₂ = elevated $p\text{CO}_2$.

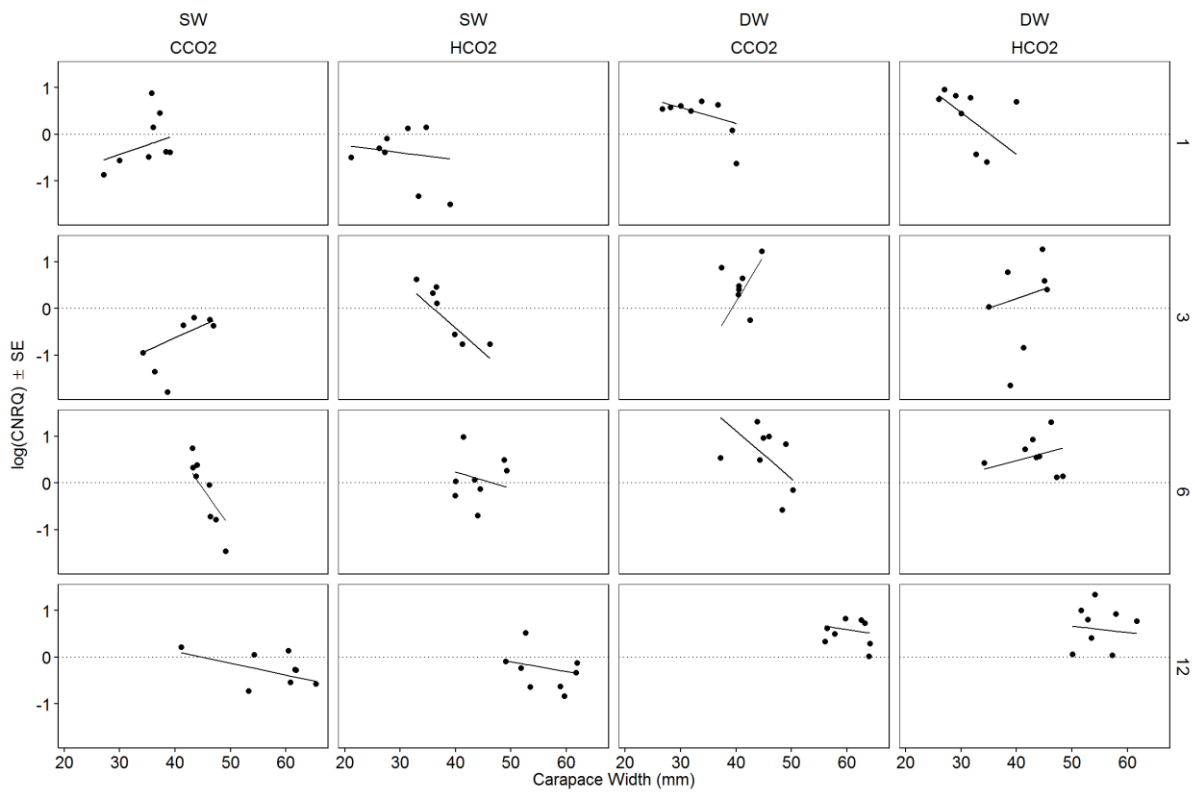


Figure S5. Relationship between *CAC* expression in the posterior gills of *Carcinus maenas* and body size given as carapace width in all 4 factor combinations: SW/control $p\text{CO}_2$; SW/ elevated $p\text{CO}_2$; DW/control $p\text{CO}_2$; DW/elevated $p\text{CO}_2$, where SW = seawater at a salinity of 33 and DW = dilute seawater at a salinity of 25. Gene expression shown as differences in $\log(\text{CNRQ})$. $\log(\text{CNRQ})$ is plotted relative to the mean for baseline crabs (horizontal broken lines). Straight, solid lines represent fits of the best model (Table 2).

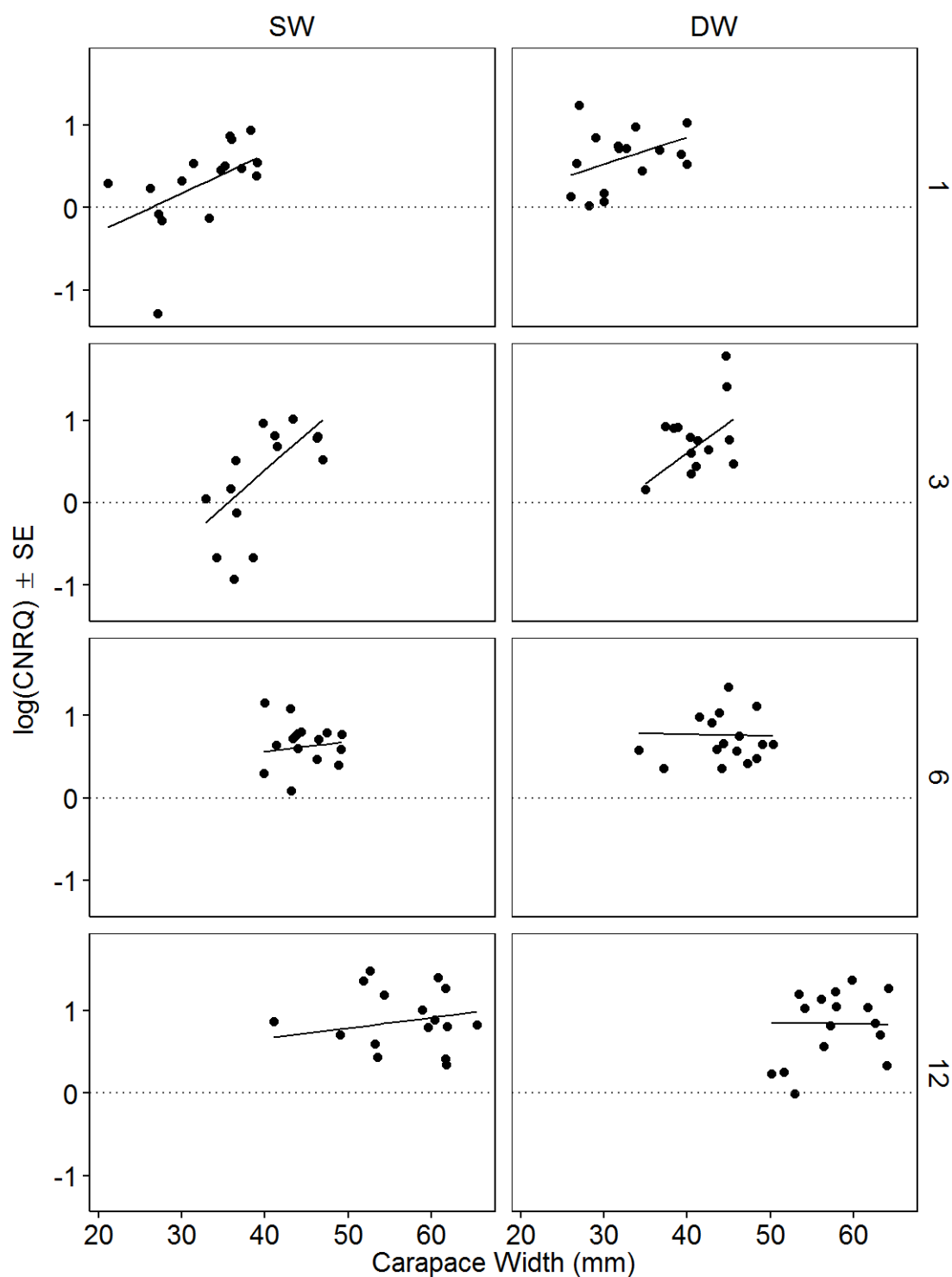


Figure S6. Two-way interactions between body size and time, and body size and salinity for *NHE* expression in the posterior gills of *Carcinus maenas*. SW = control salinity at 33, and DW = dilute seawater at 25. Time given as 1, 3, 6 and 12 sampling months. Log(CNRQ) is plotted relative to the mean for baseline crabs (horizontal broken lines). Straight, solid lines represent fits of the best model (Table 2). Overall, body size had more of an effect on *NHE* expression at one and 3 months, with a decrease in *NHE* expression in smaller crabs in DW.

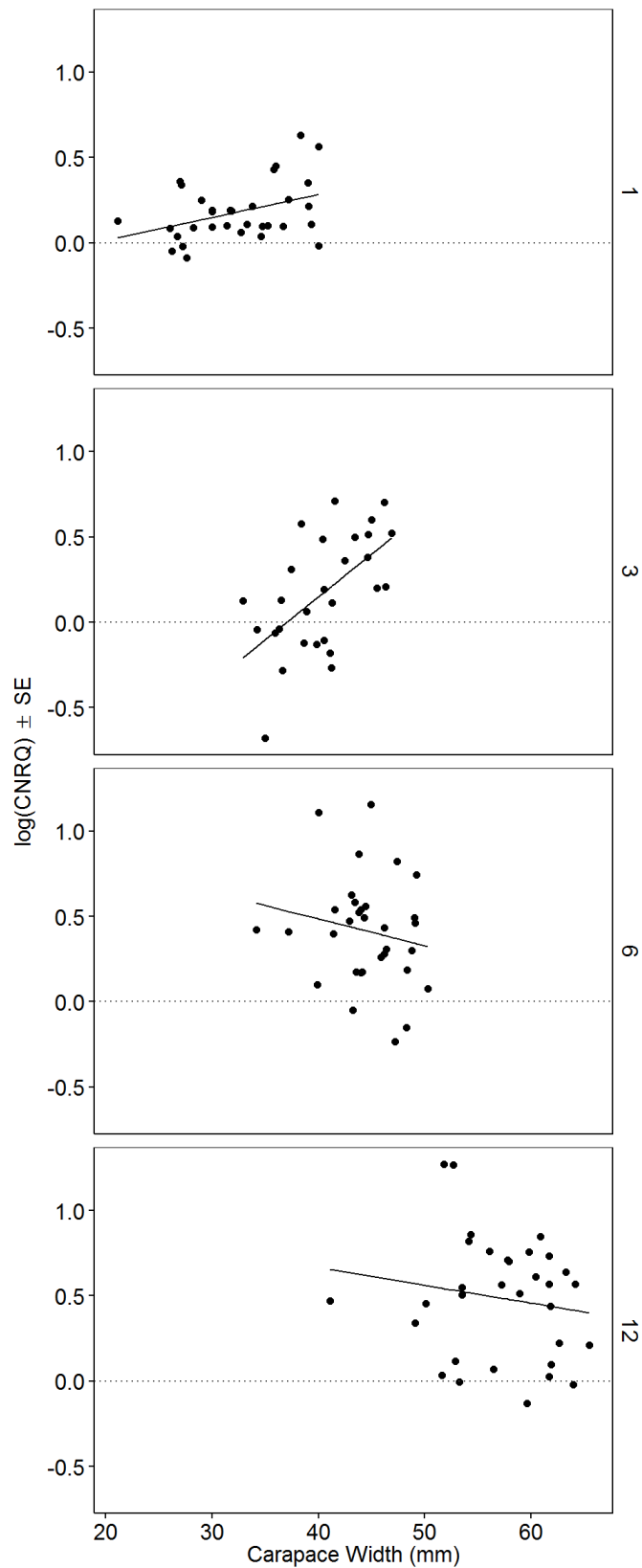


Figure S7. Relationship between *AE* expression in the posterior gills of *Carcinus maenas* and body size given as carapace width and time as 1, 3, 6 and 12 months. $\log(\text{CNRQ})$ is plotted relative to the mean for baseline crabs (horizontal broken lines). Straight, solid lines represent fits of the best model (Table 2).

Table S8. AICc values and outcomes from model selection for gene expression (log (CNRQ)) in *Cancer pagurus*.

Full model	Term removed	<i>AE</i> AICc	<i>CAC</i> AICc	<i>gpi-CA</i> AICc	<i>NKAα</i> AICc	<i>NHE</i> AICc	<i>VATB</i> AICc
4-way factorial		305.0	109.13	128.26	81.15	na	67.96
	CW:Sal:pCO ₂ :Time	297.8	99.13	123.96	82.54 ^{b,c}	na	61.16
3-way factorial		297.8	99.13	123.96	82.54	na	61.16
	Sal:pCO ₂ :Time	293.3	90.15	118.51	75.02	na	51.40
3-way terms with CW		293.3	90.15	118.51	75.02	na	51.40
	CW:pCO ₂ :Time	284.1	83.51	109.28	67.40	na	43.05
	CW:Sal:Time	284.4	81.42	110.71	74.12	na	44.93
	CW:Sal:pCO ₂	291.6	87.15	118.61 ^a	73.66	na	48.30
2-way factorial		274.3	73.71	101.92	64.21	na	34.32
	pCO ₂ :Time	266.5	66.44	93.98	56.83	na	27.82
	Sal:pCO ₂	271.5	71.80	99.14	61.66	na	32.33
	Sal:Time	267.2	68.34	94.07	58.52	na	26.61
2-way with CW		257.1	59.98	83.96	49.23	na	18.54
	CW:Time	251.9	53.51	80.96	44.17	na	13.10
	CW:Sal	255.8	57.50	82.68	48.11	na	16.93
	CW:pCO ₂	254.8	57.48	81.60	46.74	na	16.09
Additive		248.0	48.91	76.88	41.41	na	9.02
	Sal	245.7	47.12	75.10	39.15	na	16.49
	pCO ₂	249.6 ^{b,d}	46.82	75.86	39.18	na	7.53
	Time	245.3	55.62	70.92	34.71	na	20.32
	CW	248.6 ^a	46.71	77.50 ^a	41.33	na	14.19

The fourth order interaction was significant for *NKAα* although the resulting increases in AICc were relatively small (<2). No terms were retained in the model after removal of this 4-way interaction. In all cases, additional simplification resulted in models with consistently lower AICc values than the complex models.

a Removed because log-likelihood ratio test not significant (alpha = 0.05)

b Log-likelihood ratio test significant (alpha = 0.05)

c interaction removed to explore simpler models

d Could be removed from the best model (AICc for pCO₂ only model = 247.0; AICc without pCO₂ = 247.7, LR test not significant (alpha = 0.05)).

Bold text: terms retained in the final model