Supplementary materials

Complex Cd–Pb Multigenerational Exposure Improves the Growth and Food Utilization of the Cutworm *Spodoptera litura*

Meifang Zhang [1,2,3,†], Jin Chen [1,2,3,4,†], Kaixuan Wu [1,2,3], Shiru Chen [1,2,3], Jianwu Wang [1,2,3] and Yinghua Shu [1,2,3,*]

1 Key Laboratory of Agro-Environment in the Tropics, Ministry of Agriculture, South China Agricultural University, Guangzhou 510642, China;

2 Guangdong Engineering Research Centre for Modern Eco-Agriculture, Guangzhou 510642, China

3 Department of Ecology, College of Natural Resources and Environment, South China Agricultural

University, Guangzhou 510642, China

4 School of Life Sciences, Guizhou Normal University, Guiyang 550025, China

* Correspondence: shuyinghua@scau.edu.cn (Y.S.)

† These authors contributed equally to this work.

Text S1: The rearing conditions of *S. litura*

The insects were reared under constant conditions of 27±1°C, 65±5% relative humidity and a 12 h dark/12 h light photoperiod in a climatic chamber. Pupae and adults were kept under the same conditions. All bioassays were carried out under the same conditions.

Text S2: *S. litura* bioassay with complex Cd–Pb exposure for the first, fifth and tenth generations

In brief, about 200 eggs were placed in a sterilized Petri dish (12 cm diameter). Following hatching, fresh dietary-food with different heavy metal concentrations were introduced daily to the Petri dish. When the larvae had grown to the second instar, larvae with a similar size and weight (0.0035–0.004 g) from every treatment were chosen and transferred to cylindrical plastic boxes (3 cm diameter, 3 cm height) after measuring the initial weight to rear individually with adequate artificial diets. Fifty individuals were used for one bioassay and each set of bioassays was repeated in triplicate. The survival rates of *S. litura* at different development stages were calculated as follows: the survival rate of the larvae = (the number of sixth instar larvae/50) × 100%; pupation rate = (number of pupae/number of the sixth instar larvae) × 100%, the pupa was complete with the tail twisting gently; and eclosion rate = (number of adults/number of pupae) × 100%, adult emerges from the pupa with its wings fully intact and flapping gently [46]. The bodies of *S. litura* at different developmental stages (larvae, pupae, and adults) were weighed individually on an electronic balance (Sartorius, No. BS 124 S, Sartorius Scientific Instruments Co., Ltd., Beijing, China).

Text S3: Determination of heavy metal concentrations in different samples

The samples, including larval body, prepupae, pupae, adults, feces, exuviates, puparium and artificial diets were vacuum dried at 60 °C for 48 h. Then, 100 mg of the above samples (dry weight) was digested using boiling nitric acid (65%) and concentrated perchloric acid. After being digested thoroughly and filtered, the clear solution was transferred to a volumetric flask, which was then filled with 20 mL of deionized water. The Cd and Pb concentrations in samples were measured by using a flame atomic absorption spectrophotometer (AAS) (HITACHI, Japan). The heavy metal concentrations in the samples were calculated as follows: concentrations of heavy metal (mg/kg) = (C × 20)/W, where C represents the Cd or Pb concentration detected by AAS and W represents the dry weight of the sample. Cd or Pb ingestion, excretion, and accumulation by *S. litura* at different developmental stages were calculated as follows: Cd or Pb ingestion (mg) = the dry weight of food ingestion × Cd or Pb concentration in dry diets/1000; Cd or Pb excretion via feces or exuviates (mg) = the dry weight of feces or exuviates) × Cd or Pb concentrations in dry feces or exuviates/1000; Cd or Pb accumulation (mg) = dry weight of insect body × Cd or Pb concentrations in insect body/1000.

Supplementary Table S1: The ingredients of artificial diets.

Ingredients	content (%)
soy powder	44.5
wheat bran	26.7
yeast exact powder	17.8
agar	7
L-ascorbic acid	1.8
Nipagin	0.9
sorbic acid	0.9
cholesterol	0.4

Supplementary Table S2: Formulae for calculating the life parameters and nutritional indices of *S. litura.*

Indicator parameters		Formulae
Survival rate	Larval survival rate (%)	(The number of sixth instar larvae/50) × 100%
	Pupation rate (%)	(The number of pupae/number of the sixth instar larvae) × 100%
	Eclosion rate (%)	(The number of adults/number of pupae) × 100%,

Nutritional indices	RCR (g/g/d)	Dry weight of food eaten/(duration of feeding (d) × mean dry weight of the larvae during the feeding period)
	AD (%)	100 × (dry weight of food eaten-dry weight of feces produced)/dry weight of food eaten
	ECI (%)	100 × dry weight gain of larva/dry weight of food eaten
	ECD (%)	100 × dry weight gain of larvae/(dry weight of food eaten–dry weight of feces produced)
	RGR (g/g/d)	Dry weight gain of the larvae during period/(duration of feeding (d) × mean dry weight of the larvae during the period)

Supplementary Table S3: Index evaluation system.

Decision goal	Survival			Weight			Food utilization				
The weight values of decision goal	0.36			0.29			0.35				
Element	Larval survival	Pupation rate	Eclosion rate	Larval weight	Pupal weight	Adult weight	AD	ECI	ECD	RCR	RGR
The weight values of element [a]	0.13	0.13	0.13	0.10	0.10	0.10	0.07	0.07	0.07	0.07	0.07

[a] The results measured were assigned scores, respectively, and then averaged.

Supplementary Table S4: The scoring criteria of indicators.

One-way ANOVA-significant differences in letter marking results		Score
Positive indicators	Negative indicators	
a	c	5
ab	bc	4
b	b	3
bc	ab	2
c	a	1

Supplementary Table S5: Interaction of treatment and generation on life history traits and food utilization of *S. litura.*

	Parameters	GLM-analysis					
		Generations (G)		Treatments (T)		*G* and *T*	
Life history traits	Larval survival rate	*F*=24.33	*P*<0.0001	*F*=9.892	*P*=0.02	*F*=14.17	*P*=0.015
	Pupation rate	***F*=5.276**	***P*=0.071**	***F*=3.324**	***P*=0.344**	***F*=8.766**	***P*=0.119**
	Eclosion rate	***F*=2.666**	***P*=0.264**	*F*=13.251	*P*=0.004	***F*=7.831**	***P*=0.166**
	Larval weight	*F*=36.973	*P*<0.0001	*F*=19.650	*P*<0.0001	*F*=31.935	*P*<0.0001
	Pupal weight	*F*=46.455	*P*<0.0001	*F*=26.024	*P*<0.0001	*F*=14.082	*P*=0.015
	Adult weigh	*F*=34.788	*P*<0.0001	*F*=19.269	*P*<0.0001	*F*=15.503	*P*=0.008
Food utilization	RCR	*F*=194.978	*P*<0.0001	*F*=18.224	*P*<0.0001	***F*=9.604**	***P*=0.087**
	RGR	*F*=301.978	*P*<0.0001	*F*=404.101	*P*<0.0001	*F*=780.439	*P*<0.0001
	ECI	*F*=371.906	*P*<0.0001	*F*=23.921	*P*<0.0001	***F*=10.558**	***P*=0.061**
	AD	*F*=294.923	*P*<0.0001	*F*=9.454	*P*=0.024	*F*=13.144	*P*=0.022
	ECD	*F*=432.243	*P*<0.0001	*F*=20.951	*P*<0.0001	*F*=12.760	*P*=0.026

Supplementary Table S6: Interaction of heavy metal treatment and generation on Pb or Cd concentrations in different samples.

Heavy metal/sample		GLM-analysis					
		Generations (G)		Treatments (T)		G and T	
Cd concentrations	Larvae	*F*=93.517	*P*<0.0001	*F*=1246.64	*P*<0.0001	*F*=53.026	*P*<0.0001
	Prepupae	*F*=185.851	*P*<0.0001	*F*=1887.705	*P*<0.0001	*F*=116.013	*P*<0.0001
	Pupae	*F*=77.798	*P*<0.0001	*F*=2535.905	*P*<0.0001	*F*=54.074	*P*<0.0001
	Adults	*F*=640.720	*P*<0.0001	*F*=2426.681	*P*<0.0001	*F*=1153.769	*P*<0.0001
	Larval feces	*F*=1159.685	*P*<0.0001	*F*=17499.104	*P*<0.0001	*F*=532.492	*P*<0.0001
	Larval exuviates	*F*=394.331	*P*<0.0001	*F*=1007.585	*P*<0.0001	*F*=189.538	*P*<0.0001
	Prepupal exuviates	*F*=38.860	*P*<0.0001	*F*=857.691	*P*<0.0001	*F*=86.378	*P*<0.0001
	Puparium	*F*=845.291	*P*<0.0001	*F*=868.025	*P*<0.0001	*F*=1003.757	*P*<0.0001
Pb concentrations	Larvae	*F*=64.274	*P*<0.0001	*F*=3124.72	*P*<0.0001	*F*=60.530	*P*<0.0001
	Pre-pupae	*F*=47.283	*P*<0.0001	*F*=1480.658	*P*<0.0001	*F*=27.067	*P*<0.0001
	Pupae	*F*=101.806	*P*<0.0001	*F*=3728.56	*P*<0.0001	*F*=73.053	*P*<0.0001
	Adults	***F*=4.556**	***P*=0.103**	*F*=973.735	*P*<0.0001	*F*=39.968	*P*<0.0001
	Larval feces	*F*=1619.764	*P*<0.0001	*F*=83760.407	*P*<0.0001	*F*=824.545	*P*<0.0001
	Larval exuviates	*F*=86.522	*P*<0.0001	*F*=449.957	*P*<0.0001	*F*=58.031	*P*<0.0001
	Pre-pupal exuviates	*F*=63.342	*P*<0.0001	*F*=2888.501	*P*<0.0001	*F*=161.464	*P*<0.0001
	Puparium	*F*=18.746	*P*<0.0001	*F*=869.986	*P*<0.0001	*F*=13.289	*P*=0.004

Supplementary Table S7: Cd accumulation in *S. litura* at different development stages from the first, fifth and tenth generations.

Insect body	Generation	Treatment			
		CK	M	Cd	**One-way ANOVA**
Sixth instar Larvae	The first generation	0.01±0.001bC	1.97±0.16aA	1.2±0.16cB	***F*=55.817, *P*<0.0001**
	The fifth generation	0.06±0.01aC	2.2±0.06aA	1.85±0.12bB	***F*=235.74, *P*<0.0001**
	The tenth generation	0.03±0.002bC	2.3±0.13aB	2.97±0.21aA	***F*=114.995, *P*<0.0001**
	One-way ANOVA	***F*=41.924 *P*<0.0001**	*F*=1.851 *P*=0.199	***F*=28.495 *P*<0.0001**	
Pre-pupae	The first generation	0.02±0.001bB	2.26±0.16abA	2.49±0.15bA	***F*=116.874, *P*<0.0001**
	The fifth generation	0.11±0.01aB	2.08±0.11bA	1.91±0.16cA	***F*=89.018, *P*<0.0001**
	The tenth generation	0.02±0.001bC	2.63±0.09aB	3.54±0.14aA	***F*=337.956, *P*<0.0001**
	One-way ANOVA	***F*=109.141 *P*<0.0001**	***F*=5.042 *P*=0.026**	***F*=29.059 *P*<0.0001**	
Pupae	The first generation	0.03±0.003bB	2.7±0.16cA	2.68±0.06bA	***F*=442.676, *P*<0.0001**
	The fifth generation	0.04±0.004aC	3.56±0.2bA	1.96±0.08cB	***F*=197.167, *P*<0.0001**
	The tenth generation	0.01±0.001cC	5.08±0.26aA	3.02±0.05aB	***F*=270.83, *P*<0.0001**
	One-way ANOVA	***F*=24.249 *P*<0.0001**	***F*=24.956 *P*<0.0001**	***F*=69.002 *P*<0.0001**	
Adults	The first generation	0.05±0.01bB	0.3±0.05aB	5.24±0.42aA	***F*=214.649, *P*<0.0001**
	The fifth generation	0.07±0.005aB	0.36±0.03aB	2.68±0.21bA	***F*=139.558, *P*<0.0001**
	The tenth generation	0.01±0.001cC	0.26±0.03aB	0.46±0.04cA	***F*=66.683, *P*<0.0001**
	One-way ANOVA	***F*=22.001 *P*<0.0001**	*F*=2.721 *P*=0.114	***F*=112.185 *P*<0.0001**	

Values were the means± the standard error. Values in the same line followed by the different capital letters were significant at the 5% level among different treatments. Values in the same row followed by the different lowercase letters were significant at the 5% level among different generations.

Supplementary Table S8: Cd excretion in *S. litura* at different development stages from the first, fifth and tenth generations.

Excretion	Generation	Treatment			
		CK	M	Cd	**One-way ANOVA**
Larval feces	The first generation	0.01±0.001cB	1.04±0.11cA	1.29±0.15bA	***F*=41.109, *P*<0.0001**
	The fifth generation	0.05±0.01aB	2.5±0.25aA	2.49±0.13aA	***F*=78.030, *P*<0.0001**
	The tenth generation	0.02±0.001bB	1.78±0.09bA	2.29±0.48aA	***F*=17.833, *P*<0.0001**
	One-way ANOVA	***F*=23.940 *P*<0.0001**	***F*=19.766 *P*<0.0001**	*F*=4.632 *P*=0.032	
Larval exuviates	The first generation	0.0004±0.0001cB	0.0063±0.002bAB	0.009±0.003bA	*F*=4.229, *P*=0.041
	The fifth generation	0.0008±0.0001bB	0.0311±0.002aA	0.0309±0.005aA	***F*=34.196, *P*<0.0001**
	The tenth generation	0.0011±0.0001aC	0.0344±0.006aA	0.0146±0.003bB	***F*=21.308, *P*<0.0001**
	One-way ANOVA	***F*=13.915 *P*=0.001**	***F*=17.248 *P*<0.0001**	***F*=9.920 *P*=0.003**	
Pre-pupal exuviates	The first generation	0.0016±0.0001bC	0.0161±0.001bB	0.0234±0.002bA	***F*=59.431, *P*<0.0001**
	The fifth generation	0.0038±0.0001aB	0.0178±0.001bA	0.0169±0.001cA	***F*=158.605, *P*<0.0001**
	The tenth generation	0.0002±0cB	0.0272±0.002aA	0.0319±0.002aA	***F*=92.24, *P*<0.0001**
	One-way ANOVA	***F*=1016.043 *P*<0.0001**	***F*=11.681 *P*=0.002**	***F*=21.682 *P*<0.0001**	
Puparium	The first generation	0.002±0.0002cB	1.51±0.24aA	0.07±0.02bB	***F*=38.372, *P*<0.0001**
	The fifth generation	0.005±0.001bB	0.44±0.06bA	0.06±0.01bB	***F*=74.874, *P*<0.0001**
	The tenth generation	0.01±0.001aC	1.35±0.26aB	2.24±0.3aA	***F*=23.885, *P*<0.0001**
	One-way ANOVA	***F*=14.772 *P*=0.001**	*F*=4.749 *P*=0.039	***F*=34.69 *P*<0.0001**	

Values were the means± the standard error. Values in the same line followed by the different capital letters were significant at the 5% level among different treatments. Values in the same row followed by the different lowercase letters were significant at the 5% level among different generations.

Supplementary Table S9: Pb accumulation in *S. litura* at different development stages from the first, fifth and tenth generations.

Insect body	Generation	Treatment			
		CK	M	Pb	**One-way ANOVA/ T-test**
Sixth instar larvae	The first generation	0.04±0.003bB	3.46±0.29aA	3.07±0.21A	***F*=82.726, *P*<0.0001**
	The fifth generation	0.07±0.01aB	4.03±0.11aA	4.08±0.24A	***F*=230.553, *P*<0.0001**
	The tenth generation	0.06±0.004a	4.1±0.23a	N.D.	***t*=-17.596, *P*<0.0001**
	One-way ANOVA /T-test	***F*=13.556 *P*=0.001**	*F*=2.551 *P*=0.119	***t*=-3.152 *P*=0.014**	
Pre-pupae	The first generation	0.08±0.005aB	8.37±0.59aA	9.34±0.66A	***F*=99.364, *P*<0.0001**
	The fifth generation	0.06±0.004bC	4.77±0.26bB	5.63±0.33A	***F*=151.02, *P*<0.0001**
	The tenth generation	0.04±0.001c	5.9±0.21b	N.D.	***t*=-27.963, *P*<0.0001**
	One-way ANOVA /T-test	***F*=32.038 *P*<0.0001**	***F*=22.034 *P*<0.0001**	***t*=5.032 *P*=0.001**	
Pupae	The first generation	0.13±0.02aB	9.17±0.56bA	10.26±1.25A	***F*=59.547, *P*<0.0001**
	The fifth generation	0.06±0.01bB	6.39±0.36cA	6.09±0.52A	***F*=95.016, *P*<0.0001**
	The tenth generation	0.03±0.003b	10.85±0.56a	N.D.	***t*=-19.233, *P*<0.0001**
	One-way ANOVA /T-test	***F*=27.876 *P*<0.0001**	***F*=23.258 *P*<0.0001**	***t*=3.342 *P*=0.012**	
Adults	The first generation	0.03±0.001bB	3.03±0.12aA	2.73±0.14A	***F*=301.569, *P*<0.0001**
	The fifth generation	0.06±0.01aC	1.79±0.28bB	3.28±0.19A	***F*=104.635, *P*<0.0001**
	The tenth generation	0.06±0.003a	2.07±0.27b	N.D.	***t*=-7.391, *P*=0.002**
	One-way ANOVA /T-test	***F*=17.699 *P*<0.0001**	***F*=6.604 *P*=0.017**	*t*=-2.155 *P*=0.068	

Values were the means± the standard error. Values in the same line followed by the different capital letters were significant at the 5% level among different treatments. Values in the same row followed by the different lowercase letters were significant at the 5% level among different generations. N.D. means none detected.

Supplementary Table S10: Pb excretion in *S. litura* at different development stages from the first, fifth and tenth generations.

Excretion	Generation	Treatment (10^{-3} mg)			
		CK	M	Pb	**One-way ANOVA / T-test**
Larval feces	The first generation	0.03±0.002bB	4.72±0.48bA	4.51±0.28A	***F*=69.039 *P*<0.0001**
	The fifth generation	0.05±0.01aC	8.91±0.88aA	6.21±1.18B	***F*=28.485 *P*<0.0001**
	The tenth generation	0.03±0.001b	6.6±0.34b	N.D.	***t*=-19.088 *P*<0.0001**
	One-way ANOVA /T-test	***F*=6.609 *P*=0.012**	***F*=11.803 *P*=0.001**	*t*=-1.398 *P*=0.228	
Larval exuviates	The first generation	0.0009±0.0003cB	0.0142±0.005bAB	0.0372±0.013A	***F*=5.336 *P*=0.022**
	The fifth generation	0.0019±0.0001bB	0.0694±0.005aA	0.0603±0.006A	***F*=74.869 *P*<0.0001**
	The tenth generation	0.0028±0.0003a	0.065±0.011a	N.D.	***t*=-5.872 *P*=0.004**
	One-way ANOVA /T-test	***F*=16.759 *P*=0.001**	***F*=17.592 *P*<0.0001**	*t*=-1.647 *P*=0.138	
Pre-pupal exuviates	The first generation	0.003±0.0001aC	0.0262±0.002cB	0.0567±0.002A	***F*=212.888 *P*<0.0001**
	The fifth generation	0.0022±0bB	0.0434±0.002bA	0.0467±0.003A	***F*=161.42 *P*<0.0001**
	The tenth generation	0.0013±0.0001c	0.0615±0.006a	N.D.	***t*=-10.756 *P*<0.0001**
	One-way ANOVA /T-test	***F*=69.828 *P*<0.0001**	***F*=22.417 *P*<0.0001**	***t*=2.981 *P*=0.018**	
Puparium	The first generation	0.04±0.01aB	3.18±0.5aA	2.48±0.47A	***F*=21.974 *P*<0.0001**
	The fifth generation	0.03±0.01aB	0.84±0.11bA	1.02±0.13A	***F*=30.644 *P*<0.0001**
	The tenth generation	0.01±0.001b	2.07±0.41ab	N.D.	***t*=-4.494 *P*=0.003**
	One-way ANOVA /T-test	***F*=14.433 *P*=0.001**	***F*=6.594 *P*=0.017**	***t*=3.351 *P*=0.012**	

Values were the means± the standard error. Values in the same line followed by the different capital letters were significant at 5% level among different treatments. Values in the same row followed by the different lowercase letters were significant at 5% level among different generations. N.D. means N.D. means none detection.

Supplementary Table S11: Interaction of treatment and generation on Cd or Pb ingestion, accumulation, and excretion by *S. Litura.*

Heavy metal ingestion, accumulation, and excretion		GLM-analysis					
		Generations (G)		Treatments (T)		G & T	
Cd ingestion		$F=185.724$	$P<0.0001$	$F=1109.45$	$P<0.0001$	$F=154.336$	$P<0.0001$
Cd accumulation	Sixth instar larvae	$F=64.134$	$P<0.0001$	$F=723.321$	$P<0.0001$	$F=78.170$	$P<0.0001$
	Prepupae	$F=72.442$	$P<0.0001$	$F=1149.727$	$P<0.0001$	$F=73.535$	$P<0.0001$
	Pupae	$F=214.466$	$P<0.0001$	$F=2542.577$	$P<0.0001$	$F=214.514$	$P<0.0001$
	Adults	$F=303.181$	$P<0.0001$	$F=1223.643$	$P<0.0001$	$F=519.30$	$P=0.007$
Cd excretion	Larval feces	$F=67.944$	$P<0.0001$	$F=419.028$	$P<0.0001$	$F=47.583$	$P<0.0001$
	Larval exuviates	$F=47.206$	$P<0.0001$	$F=116.802$	$P<0.0001$	$F=42.273$	$P<0.0001$
	Pre-pupal exuviates	$F=56.871$	$P<0.0001$	$F=566.515$	$P<0.0001$	$F=69.553$	$P<0.0001$
	Puparium	$F=239.422$	$P<0.0001$	$F=196.29$	$P<0.0001$	$F=277.721$	$P<0.0001$
Pb ingestion		$F=158.171$	$P<0.0001$	$F=912.697$	$P<0.0001$	$F=117.124$	$P<0.0001$
Pb accumulation	Sixth instar larvae	$F=9.579$	$P=0.008$	$F=624.84$	$P<0.0001$	**$F=5.018$**	**$P=0.171$**
	Pre-pupae	$F=111.87$	$P<0.0001$	$F=851.109$	$P<0.0001$	$F=58.234$	$P<0.0001$
	Pupae	$F=99.287$	$P<0.0001$	$F=1282.88$	$P<0.0001$	$F=77.587$	$P<0.0001$
	Adults	**$F=1.556$**	**$P=0.459$**	$F=150.584$	$P<0.0001$	**$F=1.063$**	**$P=0.786$**
Pb excretion	Larval feces	$F=24.07$	$P<0.0001$	$F=409.511$	$P<0.0001$	$F=18.893$	$P=0.001$
	Larval exuviates	$F=37.92$	$P<0.0001$	$F=136.866$	$P<0.0001$	$F=27.697$	$P<0.0001$
	Pre-pupal exuviates	$F=41.534$	$P<0.0001$	$F=761.98$	$P<0.0001$	$F=85.623$	$P<0.0001$
	Puparium	$F=28.202$	$P<0.0001$	$F=240.664$	$P<0.0001$	$F=16.648$	$P=0.001$

Supplementary Figure S1: Deformity of *S. litura* pupae from the 8th generation under continuous Pb stress