Lethal effects of Cr(III) alone and in combination with propiconazole and clothianidin in honey bees

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S1. Colony variability analysis

Table S1. Results of repeated-measures rank-transformed ANOVA testing for differences among colonies in each treatment for the estimation of Cr LD_{50}.

Treatments	F-value	P-value
Control (0 mg Cr L^{-1})	0.175	0.684
$Cr(NO_3)_3 \cdot 9H_2O$ (514 mg Cr L^{-1})	0.019	0.894
$Cr(NO_3)_3 \cdot 9H_2O$ (1632 mg Cr L^{-1})	0.031	0.864
$Cr(NO_3)_3 \cdot 9H_2O$ (2177 mg Cr L^{-1})	0.946	0.352
$Cr(NO_3)_3 \cdot 9H_2O$ (2667 mg Cr L^{-1})	0.005	0.945
$Cr_2(SO_4)_3$ (302 mg Cr L^{-1})	0.021	0.887
$Cr_2(SO_4)_3$ (938 mg Cr L^{-1})	1.508	0.245

$Cr_2(SO_4)_3$ (1336 mg Cr L^{-1})	0.001	0.976
$Cr_2(SO_4)_3$ (1865 mg Cr L^{-1})	0.862	0.373
$Cr_2(SO_4)_3$ (2685 mg Cr L^{-1})	0.033	0.858

Table S2. Results of repeated-measures rank-transformed ANOVA testing for differences among colonies in each treatment in the binary and ternary mixture experiment.

Treatments	F-value	P-value
Negative control	0.223	0.646
Solvent control	0.323	0.581
Cr	1.326	0.274
CLO	0.97	0.346
PRO	0.02	0.891
CLO+PRO	0.005	0.944
PRO+Cr	0.009	0.925
CLO+Cr	2.165	0.169
CLO+PRO+Cr	0.074	0.791

S2. Mortality data *vs* Time

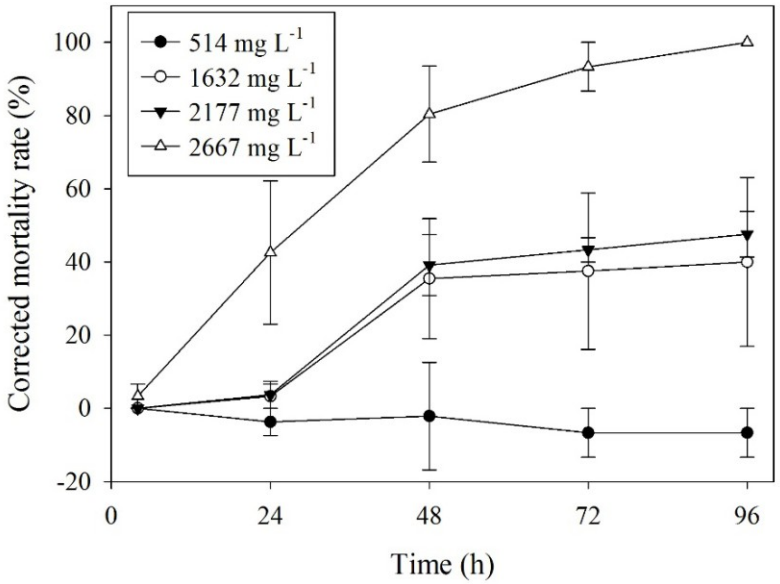

Figure S1. Corrected mortality rate with Abbott's formula: bees exposed to $Cr(NO_3)_3$.

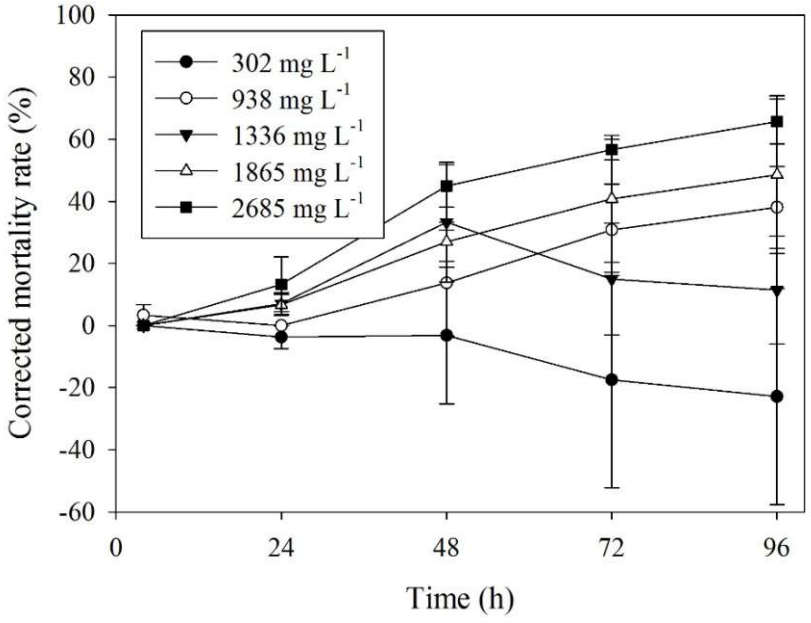

Figure S2. Corrected mortality rate with Abbott's formula: bees exposed to $Cr_2(SO_4)_3$.

S3. Regression analysis of Cr-retained and MBR data

S3.1 Cr-retained data

Visual inspection of Cr-retained data (see Figure 1a in the manuscript) indicates that the amount of Cr retained in bee bodies grows with the amount of Cr dissolved in syrup. Moreover, there is a tendency of those points to increase their dispersion as we move towards more dissolved Cr, indicative of the presence of heteroscedasticity. Consequently, we fitted those points with a generalized linear model, assuming a Gamma model distribution for the residuals and a parabolic link function for the mean response (see R script in section S3.3 of this document). Since Cr content in control bees was found below LOD, we also assumed that, in laboratory bees, retained Cr present in bee bodies must necessarily be negligible if there is no ingestion. Accordingly, we forced the parabolic link function to pass through the origin of coordinates:

(Eq. S1)

$$\mu = \alpha_{A1} \cdot Cr_{syrup} + \beta_{A1} \cdot Cr_{syrup}^2$$

where μ stands for the expected Cr retained in bee body. Table S3 shows the results of the generalized linear fit. As we can see, the coefficient α_{A1} is statistically very significant ($p<0.001$), although coefficient β_{A1} is only marginally significant at $p = 0.09$. A backward stepwise Akaike model selection procedure (function stepAIC in MASS R package), however, did not remove the quadratic term Cr_{syrup}^2 in Eq. S1.

Table S3 – Parameters of Eq. S1 calculated as explained in the text.

	Estimate	**Std. error**	**t-value**	**Pr(>|t|)**
α_{A1}	7.239×10^{-5}	1.738×10^{-5}	4.165	0.0006
β_{A1}	1.828×10^{-8}	1.013×10^{-8}	1.804	0.0880

Figure S3 displays diagnostic plots for the generalized regression, carried out with function glm.diag.plots of the boot R package. Residuals (Fig. S3a) show that very little unexplained structure remains after the fit. Furthermore, points in the QQ plot of standardized deviance residuals (Fig. S3b) are located approximately along the diagonal, indicating that the Gamma model distribution assumption was appropriate. Finally, in the two Cook's statistic (Cook 1977; Hines and Hines 1995) plots (Figs. S3 c and d), values are all smaller than the value suggested in Cook (1977),

$F(p, n-p, 0.5) = F(2,18,0.5) = 0.72$, where p stands for the number of parameters and n is the number of data points. This result signals the absence of any influential observation.

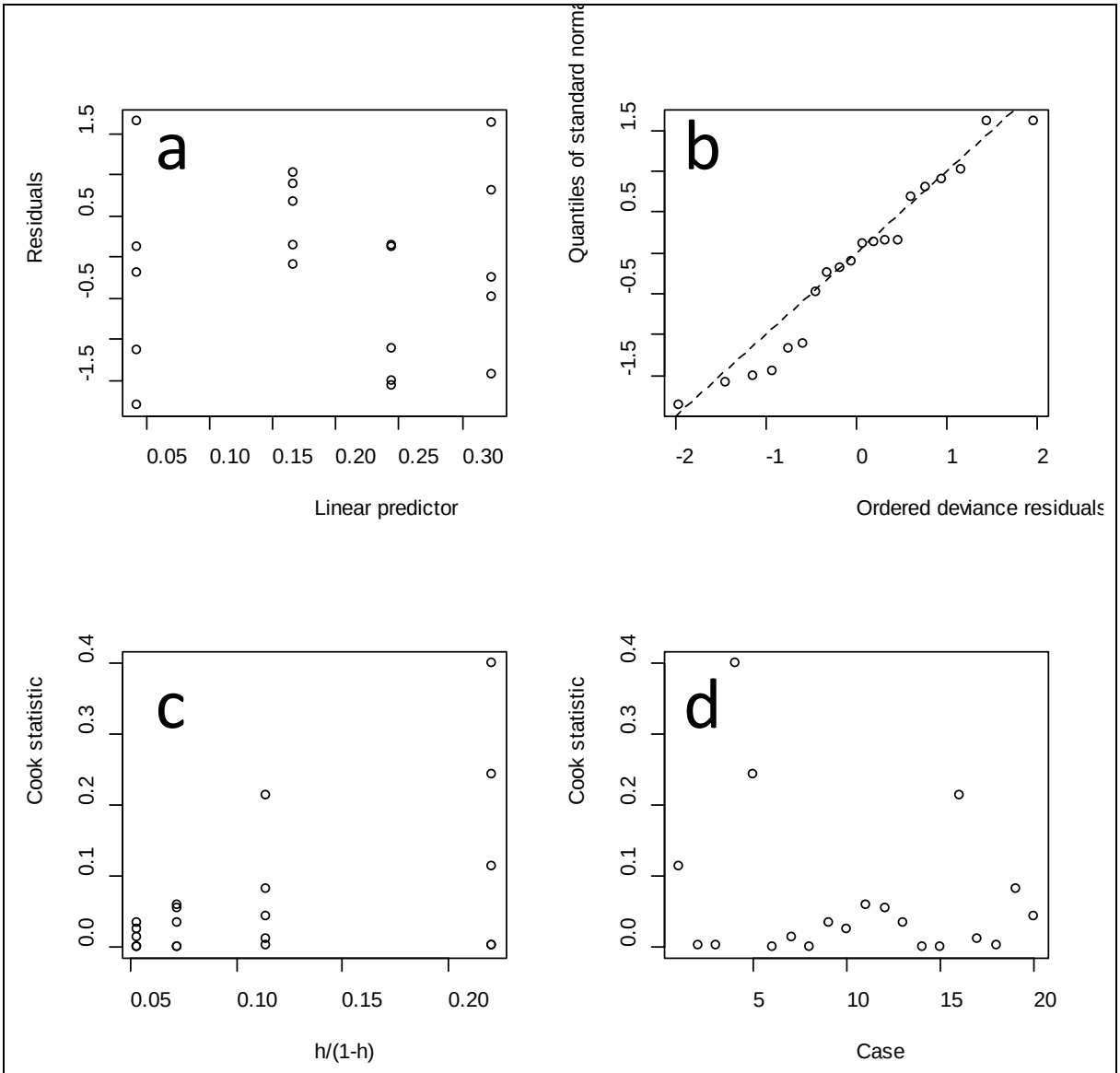

Figure S3. Diagnostic plots for the generalized linear regression of Eq. S1. From top to bottom and from left to right: a) jackknife deviance residuals vs. predictor; b) QQ plot of standardized deviance residuals; c) Cook's statistic vs. standardized leverage; and d) Cook's statistic vs. observation number.

S3.2 MBR data

An examination of the MBR data (see Figure 1b in the manuscript) shows an apparent increment of MBR with the concentration of Cr ingested by the bees. There is no clear dependence of data dispersion on the fitted values, thus discarding the existence of heteroscedasticity. In addition, the

general trend of the set of observational points does not show a convergence to MBR=0 when the amount of Cr in syrup approaches a zero value, as would have been expected in laboratory bees (see above). We therefore fitted by least-squares the MBR dataset with two different curves:

1. a straight line with no constraints (i.e. both intercept and slope parameters are free to vary), with the lm function of the stats R package:

(Eq. S2)

$$\mu = \alpha_{A1a} + \beta_{A1a} \cdot Cr_{syrup}$$

2. a non-linear power function without an intercept parameter such that it passes through the origin, with the nls function of the stats R package:

(Eq. S3)

$$\mu = \alpha_{A1b} \cdot Cr_{syrup}^{\beta_{A1b}}$$

The resulting curves are shown in Fig. 1b in the manuscript. The fitted straight line and non-linear curves yield very similar results for values of Cr in syrup larger than 500. However, when bees do not ingest Cr in the syrup, the straight line is clearly not a good model because it predicts an amount of Cr in bee body larger than zero. Tables S4 and S5 present the results of the two regressions. Although both curves seem to reproduce the visual trend of the data, only the intercept of Eq. S2 is statistically significant at $p = 0.05$.

Table S4 – Parameters of Eq. S2 calculated as explained in the text.

	Estimate	**Std. error**	**t-value**	**Pr(>|t|)**
α_{A1a}	1.888×10^{-1}	5.042×10^{-2}	3.744	0.0015
β_{A1a}	3.153×10^{-5}	2.623×10^{-5}	1.202	0.2449

Table S5 – Parameters of Eq. S3 calculated as explained in the text.

	Estimate	**Std. error**	**t-value**	**Pr(>|t|)**
α_{A1b}	0.0640	0.0722	0.886	0.387
β_{A1b}	0.1824	0.1514	1.205	0.244

Figure S4 shows the diagnostic plots corresponding to the non-linear regression of Eq. S3. Patterns in those plots closely follow those observed in Fig. S3. Namely, residuals (Fig. S4a) show a horizontal distribution without much structure left. In turn, the QQ plot (Fig. S4b) depicts data points that are distributed along the diagonal. Finally, the two Cook's statistic plots (Figs. S4 c and d) show that all values of this statistics are well below the 0.72 threshold.

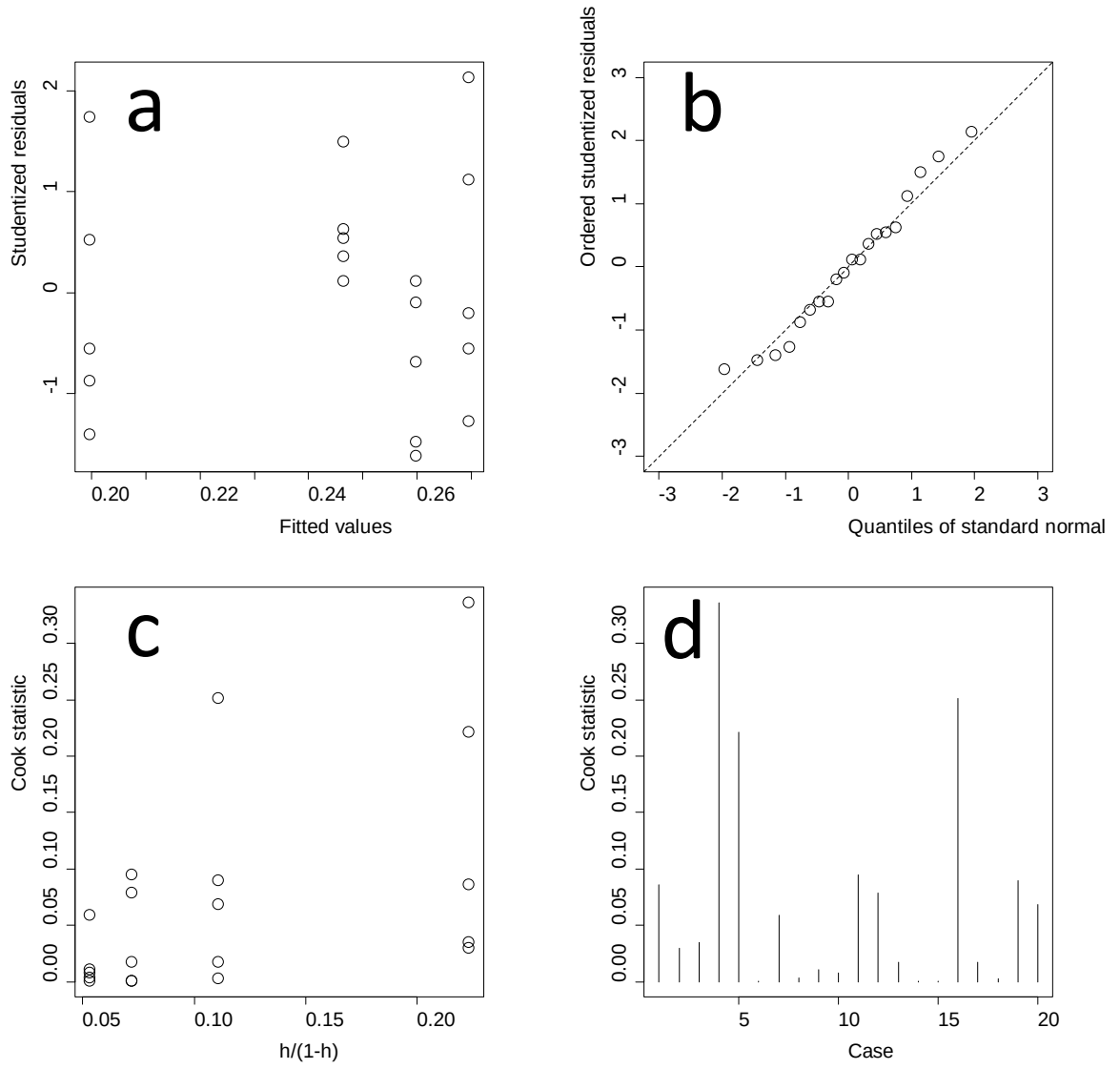

Figure S4. Diagnostic plots for the standard linear regression of Eq. S3. From top to bottom and from left to right: a) studentized residuals vs. fitted values; b) QQ plot of studentized residuals; c) Cook's statistic vs. standardized leverage; and d) Cook's statistic vs. observation number.

S3.3 – R script for regressions and diagnostics of retained Cr and MBR data as a function of Cr feeding solution.

```
# install.packages("nlstools")
# install.packages("boot")
# install.packages("MASS")
# install.packages("nlreg")
library(nlstools)
library(boot)
library(MASS)
library(nlreg)

# Dataset.
a <- read.table("Cr dataset.csv",dec=".",sep=";",header=T)

############## Cr. retained. Linear fit. Gamma-distributed residuals.
r1 <- glm(Cr.reteined.in.bee.body~-1+Cr.in.syrup+I(Cr.in.syrup^2),data=a,family=Gamma(link="identity"))
r1 <- stepAIC(r1)
win.metafile("Fig1a.emf")
plot(a$Cr.in.syrup,a$Cr.reteined.in.bee.body,ylim=c(0,.6),xlim=c(0,3000),
    xlab="Cr in syrup",ylab="Cr in bee body",pch=1,cex=2,cex.axis=1.5,cex.lab=1.5)
x <- seq(0,3000,length=1000)
points(x,predict(r1,newdata=data.frame(Cr.in.syrup=x)),type="l",lwd=2)
text(100,.55,"a",cex=3)
dev.off()
print(summary(r1))

# DIAGNOSTICS.
# - AIC model selection keeps both linear terms Cr.in.syrup and Cr.in.syrup^2.
# - Plot of residuals vs. linear predictor does not show any recognizable structure, suggesting
#   that the model is reasonable.
```

```
# - Deviance residual plot closely follows the diagonal of the plot.
# - Plots of Cook statistic (or Cook's distance) vs. h(/(1-h) or vs. Case show a maximum value
#   of ~0.4, whereas all other points are always <0.3, indicating little leverage.
win.metafile("Fig1a-diagnostics.emf")
glm.diag.plots(r1)
dev.off()

cat(paste("Threshold for Cook's statistic",qf(.5,2,20)))

############# MBR. Non-linear fit. Normal residuals.
par(mfcol=c(1,1))
r2 <- nls(MBR~c1*Cr.in.syrup^c2,data=a,start=list(c1=.3,c2=0.001))
plot(a$Cr.in.syrup,a$MBR,ylim=c(0,.6),xlim=c(0,3000),xlab="Cr in syrup",ylab="MBR",pch=1,cex=1.5)
points(x,predict(r2,newdata=data.frame(Cr.in.syrup=x)),type="l",lwd=2)
print(summary(r2))

# DIAGNOSTICS.
# - Plot of residuals vs. fitted values does show some structure still remaining, suggesting
#   that the model is reasonable but could be improved.
# - Quantile plot closely follows the diagonal of the plot.
# - Plots of Cook statistic (or Cook's distance) vs. h(/(1-h) or vs. Case show a maximum value
#   of ~0.33, whereas all other points are always <0.3, indicating little leverage.

# We use nlreg function (nlreg R package) instead of nls. Regression results are equivalent.
rr2 <- nlreg(MBR~c1*Cr.in.syrup^c2,data=a,start=list(c1=.3,c2=0.001))
win.metafile("Fig1b-diagnostics1.emf")
plot.nlreg.diag(nlreg.diag(rr2),which=2,cex=2,cex.axis=1.5,cex.lab=1.5)
dev.off()
win.metafile("Fig1b-diagnostics2.emf")
plot.nlreg.diag(nlreg.diag(rr2),which=4,cex=2,cex.axis=1.5,cex.lab=1.5)
dev.off()
win.metafile("Fig1b-diagnostics3.emf")
plot.nlreg.diag(nlreg.diag(rr2),which=6,cex=2,cex.axis=1.5,cex.lab=1.5)
```

```
dev.off()

win.metafile("Fig1b-diagnostics4.emf")

plot.nlreg.diag(nlreg.diag(rr2),which=8,cex=2,cex.axis=1.5,cex.lab=1.5)

dev.off()


############# MBR. linear fit. Normal residuals. Dose-0 requirement dropped.

r3 <- lm(MBR~Cr.in.syrup,data=a)

win.metafile("Fig1b.emf")

plot(a$Cr.in.syrup,a$MBR,ylim=c(0,.6),xlim=c(0,3000),xlab="Cr in
syrup",ylab="MBR",pch=1,cex=2,cex.axis=1.5,cex.lab=1.5)

points(x,predict(r2,newdata=data.frame(Cr.in.syrup=x)),type="l",lwd=2,lty=1)

points(x,predict(r3,newdata=data.frame(Cr.in.syrup=x)),type="l",lwd=2,lty=2)

lines(c(1800,2200),c(.55,.55),type="l",lty=1,lwd=2)

lines(c(1800,2200),c(.5,.5),type="l",lty=2,lwd=2)

text(100,.55,"b",cex=3)

text(2250,.55,"Non-linear",pos=4,cex=1.7)

text(2250,.5,"Linear",pos=4,cex=1.7)

dev.off()


# DIAGNOSTICS.

# par(mfrow=c(2,2))

# plot(r3,which=c(1,2,5,4),ask=F)

# par(mfrow=c(1,1))
```

References

Cook, R.D. (1977). Detection of Influential Observation in Linear Regression. Technometrics 19(1), 15-18.

Hines, R.J.O.H. and Hines, W.G.S. (1995). Exploring Cook's Statistic Graphically. The American Statistician 49(4), 389-394.

S4. R script for Binomial Proportional Test

```
bliss.add.test <- function(ndead,ntot,conf.level=0.05,alternative="two.sided") {
  p <- ndead/ntot
  pab.obs <- p[3]                  # Observed proportion.
  pab.exp <- p[1]+p[2]-p[1]*p[2]    # Expected proportion.
  p.dif <- pab.obs-pab.exp         # Difference. Null hyp. is p.dif=0.
  names(p.dif) <- "Observed prop. minus expected prop."
  vara <- p[1]*(1-p[1])/ntot[1]
  varb <- p[2]*(1-p[2])/ntot[2]
  varab.obs <- p[3]*(1-p[3])/ntot[3]
  varab.exp <- vara+varb+p[2]^2*vara+p[1]^2*varb    # Derived with the Delta method.
  sd.all <- sqrt(varab.obs+varab.exp)
  ci <- function(p.value) switch(alternative,two.sided=c(p.dif+sd.all*qnorm(1-p.value/2)*c(-1,1)),
                                 less=c(-Inf,p.dif+qnorm(1-p.value)*sd.all),
                                 greater=c(p.dif-qnorm(1-p.value)*sd.all,Inf))
  ff <- function(p.value) min(abs(ci(p.value)))
  p.value <- optimize(ff,interval=c(0,1))$minimum
  out <- list(method="Improved binomial proportion test for additivity",
              statistic=p.dif,
              data.name=paste(deparse(substitute(ndead)),"and",deparse(substitute(ntot))),
```

```
            p.value=unname(p.value),
            alternative=alternative,
            conf.int=ci(conf.level))
  class(out) <- "htest"
  return(out)
}
# Mortality data. Column 1 (e.g. datamort[[1]][,1]) contains the total number of individuals, labelled "N".
datamort <- list()
datamort[[1]] <- cbind(c(60,60,50),c(3,0,12),c(6,1,24),c(13,5,30),c(23,16,34),c(28,22,38)) # Apis CLO & PRO.
datamort[[2]] <- cbind(c(60,60,60),c(0,1,2),c(1,1,2),c(5,7,5),c(16,23,16),c(22,26,19))     # Apis PRO & Cr.
datamort[[3]] <- cbind(c(60,60,60),c(3,1,6),c(6,1,12),c(13,7,17),c(23,23,30),c(28,26,36))      # Apis CLO & Cr.
datamort[[4]] <- cbind(c(50,60,60),c(12,1,21),c(24,1,26),c(30,7,30),c(34,23,35),c(38,26,38))        # Apis CLO+PRO &
Cr.
datamort[[5]] <- cbind(c(60,60,60),c(2,3,21),c(2,6,26),c(5,13,30),c(16,23,35),c(19,28,38))      # Apis PRO+Cr & CLO.
datamort[[6]] <- cbind(c(60,60,60),c(6,0,21),c(12,1,26),c(17,5,30),c(30,16,35),c(36,22,38))      # Apis CLO+Cr & PRO.
for (i in 1:6) rownames(datamort[[i]]) <- c("A","B","A+B")
for (i in 1:6) colnames(datamort[[i]]) <- c("N","4h","24h","48h","72h","96h")
# Labels.
names.experiments <- c("Apis CLO & PRO",
                       "Apis PRO & Cr",
                       "Apis CLO & Cr",
                       "Apis CLO+PRO & Cr",
```

```
                "Apis PRO+Cr & CLO",
                "Apis CLO+Cr & PRO")
time.experiments <- c("4h","24h","48h","72h","96h")
# Testing for antagonism (i.e. the "less" alternative).
res.test.ant <- list()
p.values.ant <- matrix(0,6,5)     # Uncorrected p-values.
effect.size <- matrix(0,6,5)  # Effect size (i.e. observed minus expected probabilities)
rownames(p.values.ant) <- names.experiments
colnames(p.values.ant) <- paste("Antag. ",time.experiments,sep="")
rownames(effect.size) <- names.experiments
colnames(effect.size) <- time.experiments
for (i in 1:6) {
  apis <- list()
  p <- NULL
  e <- NULL
  for (j in 2:6) {
    apis[[j-1]] <- bliss.add.test(datamort[[i]][,j],datamort[[i]][,1],alternative="less")
    p <- c(p,apis[[j-1]]$p.value)
    e <- c(e,apis[[j-1]]$statistic)
  }
  names(apis) <- paste(names.experiments[i]," - ",time.experiments,sep="")
  res.test.ant[[i]] <- apis
```

```
  p.values.ant[i,] <- p
  effect.size[i,] <- e
}
names(res.test.ant) <- names.experiments
# Holm-corrected p-values.
p.correct.ant <- t(sapply(1:6,function(i) p.adjust(p.values.ant[i,],method="holm")))
row.names(p.correct.ant) <- names.experiments
# Testing for synergy (i.e. the "greater" alternative).
res.test.syn <- list()
p.values.syn <- matrix(0,6,5)     # Uncorrected p-values.
rownames(p.values.syn) <- names.experiments
colnames(p.values.syn) <- paste("Syner. ",time.experiments,sep="")
for (i in 1:6) {
  apis <- list()
  p <- NULL
  e <- NULL
  for (j in 2:6) {
    apis[[j-1]] <- bliss.add.test(datamort[[i]][,j],datamort[[i]][,1],alternative="greater")
    p <- c(p,apis[[j-1]]$p.value)
    e <- c(e,apis[[j-1]]$statistic)
  }
  names(apis) <- paste(names.experiments[i]," - ",time.experiments,sep="")
```

```
  res.test.syn[[i]] <- apis

  p.values.syn[i,] <- p

}

names(res.test.syn) <- names.experiments

# Holm-corrected p-values.

p.correct.syn <- t(sapply(1:6,function(i) p.adjust(p.values.syn[i,],method="holm")))

row.names(p.correct.syn) <- names.experiments

#----------------

p.correct.all <- t(sapply(1:6,function(i) p.adjust(c(p.values.ant[i,],p.values.syn[i,]),method="holm")))

row.names(p.correct.all) <- names.experiments
```

S5. Results of the BMD analysis

Table S6. Results of the benchmark dose (BMD) analysis for Cr following acute oral exposure to $Cr(NO_3)_3$ in *Apis mellifera* at 48 h after ingestion. AIC: Akaike information criterion; BMDL: lower confidence limit of the benchmark dose for each model; BMDU: upper confidence limit of the benchmark dose for each model. In bold the lowest and highest BMDL and BMDU values, respectively.

Fitted Model	Number of parameters	Log-likelihood	AIC	BMDL	BMDU
Null model	1	-102.35	206.70		
LMS (two-stage)	3	-83.70	173.40	**379**	939
Log-logistic	3	-83.60	173.20	541	**1670**
Weibull	3	-83.29	172.58	506	1660
Log-probit	3	-83.58	173.16	570	1650
Gamma	3	-83.47	172.94	498	1640
Logistic	2	-84.07	172.14	471	713
LVM_Exp	3	-83.16	172.32	449	1640
LVM_Hill	3	-83.23	172.46	474	1650
Full model	4	-82.07	170.14		

Table S7. Results of the benchmark dose (BMD) analysis for Cr following acute oral exposure to $Cr_2(SO4)_3$ in *Apis mellifera* at 48 h after ingestion. AIC: Akaike information criterion; BMDL: lower confidence limit of the benchmark dose for each model; BMDU: upper confidence limit of the benchmark dose for each model. In bold the lowest and highest BMDL and BMDU values, respectively.

Fitted Model	Number of parameters	Log-likelihood	AIC	BMDL	BMDU
Null model	1	-115.25	232.50		
LMS (two-stage)	3	-107.87	221.74	320	801
Log-logistic	3	-107.80	221.74	82.1	1210
Weibull	3	-107.87	221.74	64.7	1230
Log-probit	3	-107.73	221.46	102	1210
Gamma	3	-107.87	221.74	56.7	1240
LVM_Exp	3	-107.97	221.94	**43.0**	**1250**
LVM_Hill	3	-107.94	221.88	49.7	1240
Full model	4	-107.01	222.02		