

Chronic exposure to chlorpyrifos reveals two modes of action in the springtail *Folsomia candida*

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Exposure to chlorpyrifos in food affects reproduction in springtails according to two distinct toxic mechanisms.

Abstract

Organophosphates are popular insecticides, but relatively little is known about their chronic effects on ecologically relevant endpoints. In this paper, we examine a life-cycle experiment with the springtail *Folsomia candida*, exposed via food to chlorpyrifos (CPF). The results for all endpoints (survival, growth and reproduction) were analyzed using the DEBtox model. Growth was unaffected by CPF, even at concentrations causing severe effects on survival and reproduction. Model analysis suggests that CPF directly affects the process of egg production. For the short-term response (45 days), this single mode of action accurately agreed with the data. However, the full data set (120 days) revealed a dose-related decrease in reproduction at low concentrations after prolonged exposure, not covered by the same mechanism. It appears that CPF interacts with senescence by increasing oxidative damage. This assumption fits the data well, but has little consequences for the predicted response at the population level.

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1. Introduction

Organophosphates (OPs) are a family of popular broad-spectrum insecticides. Most studies on OP toxicity focus on the acute lethal toxicity of these compounds at high concentrations. OPs are thought to exert their toxicity by binding to acetylcholinesterase (AChE), inhibiting the action of this enzyme (whose function is to degrade the neurotransmitter acetylcholine), and eventually killing the organism (Fukuto, 1990). However, less is known about the chronic effects at lower concentrations on more ecologically relevant endpoints, such as growth and reproduction (Roex et al., 2003). In our opinion,

the effects on growth and reproduction can only be properly understood from the perspective of resource allocation. The theory of Dynamic Energy Budgets (DEB, Kooijman, 2000) describes how individuals acquire and utilize energy, based on a set of simple rules for metabolic organization. Toxic effects of chemicals can be viewed as a disruption of resource allocation, e.g. by increasing the maintenance costs or decreasing assimilation of energy from food. This insight forms the basis of the DEBtox method, which was developed to provide a process-based analysis of results from toxicity tests (Kooijman and Bedaux, 1996). Recently, we have demonstrated that the various endpoints (growth, reproduction and survival) in life-cycle studies can be analyzed together in a consistent mechanistic manner using this approach (Jager et al., 2004; Alda Álvarez et al., 2005).

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In DEBtox, the time course of the toxic effect is governed by the (estimated) dynamics of the concentration inside the organism. For OPs, however, the situation is more complex. Toxicity is not directly related to the body residue, but to the inhibition of the enzyme AChE, thus requiring an additional dynamic step in the calculations, accounting for the inactivation and repair of AChE (see Legierse et al., 1999). Recently, we presented a process-based model to quantify the dynamic toxic effects on survival for compounds that act through interactions with specific receptors (Jager and Kooijman, 2005). This model is based on the DEBtox hazard model (Bedaux and Kooijman, 1994), extended with a dynamic step concerning binding and release of the toxicant from the receptors. Even though the standard DEBtox model was able to describe the acute mortality patterns in fish, the receptor-based model led to more realistic parameter estimates, and revealed a similar mode of action for different OP esters (Jager and Kooijman, 2005).

Even though the acute effects at high concentrations are generally ascribed to inhibition of AChE, long-term effects of OPs may be related to other mechanisms of action. For example, the same percentage depression of AChE activity led to a much larger decrease in growth for chlorpyrifos than for diazinon in the earthworm *Aporrectodea caliginosa* (Booth and O'Halloran, 2001). An alternative candidate for the sub-lethal mechanism of action of CPF is through induction of oxidative damage, as was demonstrated in fish (Hai et al., 1997; Ozcan Oruc et al., 2004). The aim of this study was to shed more light on the chronic lethal and sub-lethal effects of OPs. For this purpose, we use a previously published data set on the effects of chlorpyrifos on the life-cycle of the springtail *Folsomia candida* (Crommentuijn et al., 1997).

2. Materials and methods

2.1. Experimental data

The data set for chlorpyrifos (CPF) in the springtail *F. candida* is taken from Crommentuijn et al. (1997). The experiments started with one-day old juveniles, and the animals were provided with CPF-spiked food (baker's yeast, spiked with 0–20 mg/kg CPF). Animals were kept individually in perspex containers, using 30 replicates for each treatment (15 animals to record weight changes, and 15 to assess egg production). Survival, body size and reproduction were recorded three times per week, for a period of 120 days.

The data set was split into a short-term set, comprising the survival and reproduction data for the first 45 days of exposure, and a long-term set (the entire 120 days). The reason for also looking at a reduced data set is that an experimental duration of 45 days is more feasible for routine applications than a full life-cycle duration of 120 days. We wanted to investigate whether a relatively short experiment provides sufficient information on the mode of action of the compound, and allows for a representative calculation of the population consequences.

2.2. Model description

The inhibition dynamics of AChE by CPF was modeled through a receptor mechanism, according to Jager and Kooijman (2005). This more elaborate model was used because it more closely represents our knowledge about the toxic mechanism, and because it was shown that it provided a better fit on the survival data for CPF in *F. candida* (Jager and Kooijman, 2005). In short: functional receptors in the organism are knocked out by the chemical, which

produces non-functional ones. The total number of receptors is a constant fraction of the body volume (thus implying a production of new functional receptors when the animal grows). Functional receptors are turned into non-functional ones by reaction with the chemical. Thus, the production rate of non-functional receptors depends on the meeting frequency between functional receptors and the molecules of the compound inside the organism. Non-functional receptors may be repaired, either by removing the inhibitor from the receptor, or by receptor turnover (replacing “old” receptors by new ones). The effects on the endpoints can now be related to the density of non-functional receptors. For mortality, the inhibited receptors simply increase the probability to die (through the hazard rate, see Jager and Kooijman, 2005) when receptor occupation exceeds a no-effect concentration (NEC). For effects on growth and reproduction, various energy-based options are available as mode of action, such as an increase of the maintenance rate, or an increase in the costs for egg production (Kooijman and Bedaux, 1996).

The long-term data set required a description of the effects of senescence on survival and reproduction. Several adaptations to the model as presented earlier (Jager et al., 2004) were applied. Firstly, the maintenance rate coefficient was no longer used as a free parameter, but linked directly to the growth rate constant (Kooijman and Bedaux, 1996). The analysis for other compounds on *F. candida* resulted in unrealistically low values of maintenance (Jager et al., 2004). However, to provide an adequate description of the old-age effects on reproduction and survival with this more realistic maintenance rate, it was necessary to relate the absolute amount of oxidative damage in the animal to an increased hazard to the adults and the developing embryos, instead of damage density. The validity of this assumption is not entirely clear and requires dedicated experimental work, but this formulation provides an adequate description of the ageing effects, without the need for additional feedback mechanisms (see also Alda Álvarez et al., 2005).

2.3. Fitting the model to the data

The differential equations were implemented in MatLab® (version 7.0.1), and solved with an ODE solver; the scripts used to calculate the model are part of the DEBtool collection that can be obtained from <http://www.bio.vu.nl/thb/deb/deblab/>. The model is fitted to the data for multiple endpoints (in this case survival and reproduction) simultaneously using maximum likelihood estimation (see Jager et al., 2004). Likelihood-based confidence intervals were generated using profile likelihoods (see Meeker and Escobar, 1995).

The initial length was fixed as reported in the original paper (0.0262 mm, volumetric length, which is the cubic root of body weight, assuming a specific density of 1 g cm⁻³). For the receptor model, the elimination rate is fixed to a high value because the reaction with the receptors likely occurs in the water phase inside the organism (Legierse et al., 1999), and this assumption seemed to fit the data well (Jager and Kooijman, 2005). An additional problem with the receptor model occurs when the number of occupied receptors remains low throughout exposure. In that case, the toxicity parameters (NECs, killing rate, tolerance concentration, and receptor knock-out rate) tend to be heavily correlated (Jager and Kooijman, 2005). A high value for the knock-out rate can simply be compensated by the other parameters. This either leads to very broad confidence intervals or unreliable ones (the profile likelihood requires multiple optimizations, which are hampered by the parameter correlations). To address these problems, one parameter of this set was fixed; the NEC for survival is set at 1% receptor occupation. This strongly helps the optimization and calculation of confidence intervals, but the intervals for the toxicity parameters must now be interpreted relative to this fixed NEC for survival.

2.4. Population effects

Intrinsic rates of population increase were calculated using the continuous form of the Euler–Lotka equation (see Jager et al., 2004), by integrating survival and reproduction over the entire exposure period where data were available (45 days for the short-term set, and 120 days for the full data set). This population growth rate was calculated both using the model predictions for survival and reproduction, as well as using the data themselves. For the latter, the survival and reproduction were interpolated on a smaller time grid (using

piecewise cubic Hermite interpolation in MatLab) and subsequently integrated in the Euler–Lotka equation. Model predictions were also made for the situation where food supply is limiting (see Jager et al., 2004).

3. Results and discussion

3.1. Growth data

CPF up to an exposure concentration of 20 mg/kg food had no effect at all on the growth pattern (Fig. 1), not even at concentrations that seriously affected survival and reproduction (Fig. 2). From a DEBtox perspective, this leaves only two modes of action: CPF either poses a direct hazard to the developing egg (i.e. induces mortality during oogenesis), or increases the costs of egg production (see Kooijman and Bedaux, 1996). Because CPF does not seem to affect individual growth curves (Fig. 1), the growth parameters were estimated on the data for all concentrations and fixed for subsequent calculations, growth rate constant 0.0580 (0.0565–0.0594) d⁻¹, maximum volumetric length 0.653 (0.649–0.658) mm (maximum likelihood estimates with 95% likelihood-based confidence intervals).

3.2. Short-term data

For survival and reproduction, we first focus on the initial part of the data (0–45 days). For this short-term data set, the results are quite clear, and a simultaneous fit of the receptor model on survival and reproduction fitted the data well (Fig. 2). Regarding the mode of action on reproduction, the direct hazard to eggs during oogenesis fitted the data best. This mode of action also makes sense intuitively; as the adults also suffer lethal effects due to AChE inhibition at these doses, it is conceivable that the developing eggs are affected in a similar fashion. Teratogenic effects of OP pesticides have been demonstrated in developing fish embryos (see e.g. Cook

et al., 2005). It should be noted that this mode of action was deduced from the lack of effects on growth and the specific pattern of reduction in the rate of egg production. More detailed histological work would be needed to validate this hypothesis.

The estimated model parameters are given in Table 1 with their confidence intervals. The no-effect concentration (NEC) for survival and reproduction is in fact quite similar, indicating perhaps a similar sensitivity for adults and developing embryos to AChE inhibition. These NECs are also directly evident from the experimental data, as the lowest three concentrations do not show appreciable effects, relative to the control (Fig. 2). The receptor repair rate is clearly larger than zero, indicating that the receptor interactions are reversible (with a half life of approximately one day). For these fits, the NEC for survival was fixed at 1% inhibition of the receptor. This is very low for AChE interactions, because mortality is generally associated with much higher inhibition levels. However, such high NEC values resulted in a poorer fit to the data. The possible explanations for this discrepancy have been extensively discussed earlier (Jager and Kooijman, 2005). The NECs based on receptor occupation can simply be recalculated back to external concentrations (derived from equations given in Jager and Kooijman, 2005):

$$NEC_{\text{external}} = \frac{\text{repair rate/knock out rate}}{1/NEC_{\text{receptor}} - 1} \quad (1)$$

For the parameter estimates in Table 1, this yields a NEC of 4.9 mg/kg_{food} for survival and of 6.0 mg/kg_{food} for reproduction.

We used the receptor-based model of DEBtox for the fits in Fig. 2, because this model is a closer representation of our knowledge of the toxic mechanism. However, the standard DEBtox models also provided a reasonable fit to the data. The data for reproduction were described with the same level of accuracy, but the fit for the survival data was slightly (but significantly) worse, especially for the initial part of the curves (see Jager and Kooijman, 2005). Nevertheless, the resulting estimates for the NECs (3.8 mg/kg for survival and 5.6 mg/kg for reproduction) are very close to the ones for the receptor model, after calculation to the external values using Eq. (1). This shows that the NEC is a robust summary statistic that does not depend on model details (as long as the model fits the data).

3.3. Long-term data

When the entire 120-day data set is used, it is evident that although the lowest three exposure levels do not show appreciable effects on the short-term, they do reveal a dose-related decrease in reproduction at a longer time scale (Fig. 3). These low-dose effects will likely also be present at the highest doses, but will be hidden by the more acute effects, ascribed to AChE inhibition. It must be realized that these effects at low concentrations cannot be described by the same mechanism as the effects at high exposures, because the

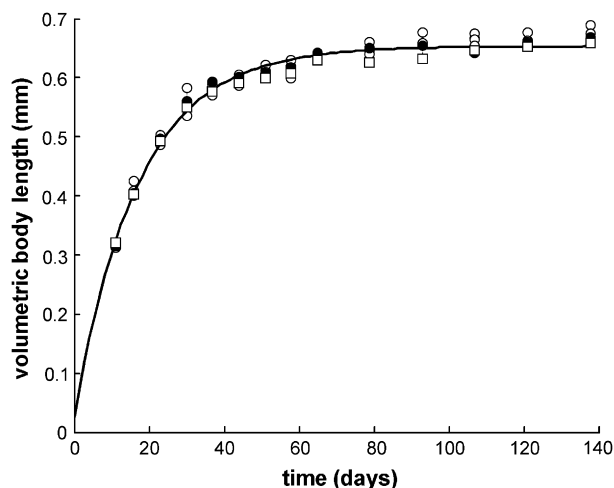


Fig. 1. Growth curves for *Folsomia candida* at chlorpyrifos concentrations ranging 0–20 mg/kg_{food} (control response shown as open squares, highest concentration as filled circles, all intermediate concentrations as open circles). Length is shown as volumetric length (third root of body volume).

Table 1

Parameter estimates with likelihood-based 95% confidence intervals for the survival and reproduction data of chlorpyrifos in growing springtails (*Folsomia candida*)

	Short-term data set (0–45 days)	Full data set (0–120 days) (simult. fit of low and high dose)	
	All doses	Low dose	High dose
Physiological parameters			
Vol. length at puberty	0.418 (0.410–0.427) mm	0.442 (0.438–0.445) mm	
Max. reproduction rate	26.2 (24.7–27.8) eggs/d	37.4 (35.3–41.4) eggs/d	
Blank hazard rate	2.17 (1.16–3.61) $\times 10^{-3}$ d ⁻¹	n.a.	
Damage killing rate	n.a.	0.920 (0.811–1.01) $\times 10^{-3}$ d ⁻¹	
Damage tolerance on repro	n.a.	10.2 (9.28–10.8) [–]	
Toxicological parameters			
Toxic mode of action	Receptor model. Direct hazard on egg production	Increase in oxidative damage related to maintenance	Receptor model. Direct hazard on egg production
Elimination rate	10 d ⁻¹ (n.e.)	2.52 (0–6.46) $\times 10^{-3}$ d ⁻¹	10 d ⁻¹ (n.e.)
No-effect concentration for survival	0.01 [–] (n.e.)	n.a.	0.01 [–] (n.e.)
Killing rate	2.92 (1.17–5.09) d ⁻¹	n.a.	3.26 (2.35–4.35) d ⁻¹
No-effect concentration for growth/repro	0.0107 (0.00800–0.0182) [–]	0 (0–0.00313) mg/kg _{food}	0.0106 (0.00941–0.0154) [–]
Tolerance concentration	6.77 (4.54–12.1) $\times 10^{-3}$ [–]	0.453 (0.00694–7.08) mg/kg _{food}	5.64 (5.32–7.40) $\times 10^{-3}$ [–]
Receptor knock-out rate	1.21 (0.681–2.59) $\times 10^{-3}$ mg kg _{food} ⁻¹ d ⁻¹	n.a.	2.50 (2.14–5.62) $\times 10^{-3}$ mg kg _{food} ⁻¹ d ⁻¹
Receptor repair rate	0.672 (0.315–1.6) d ⁻¹	n.a.	1.64 (>1.13) d ⁻¹
Growth parameters were fixed (see Section 3.1). Separate fits on the full data set (120 days) and a reduced set (45 days). n.a. is not applicable, n.e. is not estimated.			

Growth parameters were fixed (see Section 3.1). Separate fits on the full data set (120 days) and a reduced set (45 days). n.a. is not applicable, n.e. is not estimated.

toxicodynamics are quite different. The effects at the high doses occur rapidly in time, whereas the low-dose effects only occur at a much older age. Therefore, it is tempting to relate the low-dose effects to the ageing process. In DEBtox, we describe the ageing process as the result of the production of reactive oxygen species (ROS), linked to respiration (Van Leeuwen et al., 2002; Jager et al., 2004). Because OPs are known to induce oxidative stress in fish (Hai et al., 1997; Ozcan Oruc et al., 2004) and increase ROS production in cell cultures (Crumpton et al., 2000), this is a reasonable assumption. Although the CPF oxon is responsible for the AChE inhibition (Fukuto, 1990), the work of Crumpton et al. showed that it is the parent CPF that increases ROS production. Therefore, we did not have to use the receptor model for this mode of action, and assumed that CPF increases ROS production related to maintenance. With these modifications, the model was fit simultaneously to all data (growth and reproduction, at high and low doses). The low-dose data for reproduction are well described by the additional mode of action, but the description of the low-dose effects on survival is not very clear (Fig. 3). If the mechanism at low dose is indeed through an increase in oxidative damage, this should affect both reproduction and survival. However, the decrease in survival at low doses, as predicted by the model, is not really evident from the data.

The model fits for high and low doses share the same physiological parameters, but differ in the toxicological parameters (Table 1). The low-dose mode of action requires a low elimination rate, which is more in line with CPF's high hydrophobicity. The NEC for this mode of action relates both to survival and reproduction (because it deals with ROS production), and is very low (not significantly different from zero). The parameters for the high-dose receptor effects are quite similar to the results for the short-term data set, which shows that the model parameters can be accurately estimated from the short-term data alone.

3.4. Population effects

The intrinsic rate of population increase can be calculated both using the short-term data set and the long-term data set, as well as directly from the data without any model (Fig. 4). These calculations assume that all produced eggs are fertile, which was not tested. The population growth estimated directly from the data is almost exactly the same when using either the 45-day or the 120-day data. The reason for this similarity is that the early young are most important for the population growth (because of the principle of interest-upon-interest); apparently, when the animals are more than 45 days old they contribute little to the population growth rate.

The control population growth rate as predicted from the model fits is lower than that calculated directly from the data. This is caused by the fact that *F. candida* reproduces in clutches, whereas the model assumes a continuous reproduction (see Fig. 2). The model averages out these reproductive events, but because the early young contribute more to the population growth, the resulting growth rate from the

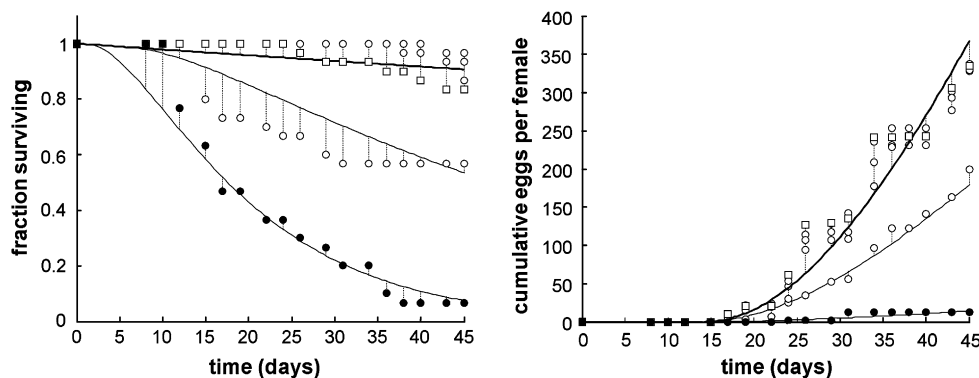


Fig. 2. Short-term survival and cumulative reproduction for *Folsomia candida* at chlorpyrifos concentrations ranging 0–20 mg/kg_{food} (control response shown as open squares, highest concentration as filled circles, all intermediate concentrations as open circles). Model fit with DEBtox using the receptor model.

model fits is somewhat lower. More interestingly, the population growth rate as calculated from the data shows a decrease at the first three exposure levels, caused by a slightly decreased reproduction in the first few reproductive events (see Fig. 2), that was not covered to that extent by the model fits. The short-term fit considers the slight decrease in reproduction to be caused by random variation (the NEC is prominently positioned around 6 mg/kg_{food}, see Fig. 4). This hypothesis provides a better fit than assuming that this decrease is caused by the chemical (and thus that the NEC is close to zero). Furthermore, the fact that the NECs for survival and reproduction are very similar (Table 1) supports this interpretation. The long-term fit does reveal a decreasing reproduction at low exposure, owing to the additional mode of action related to oxidative damage, but not to the same extent as the data. Because the long-term analysis attempts to describe the data over the entire trajectory, it can balance a poor fit after short-term exposure by good fit after long-term exposure (even though the short-term data are more important for population growth). For the highest two exposure levels, both model predictions are quite close to the estimation based on the data.

The original paper of Crommentuijn et al. (1997) also reports population growth rates calculated from the Euler–Lotka equation. However, the growth rates in that paper

are lower than the ones calculated here (the difference is approximately 0.03 d^{-1} for all concentrations). This difference probably relates to the calculation method; the earlier paper applies a discrete summation of the survival and reproduction at each experimental time point whereas we here use a more continuous integration (results are interpolated by splining the data). The considerable difference between both approaches shows that the population growth rate can be sensitive to the calculation method, especially when there are few measurements in time.

The predictions for the population growth rate are based on the conditions in the experiment, and thus on the presence of ad libitum food availability. However, food limitation is a common stressor under field conditions, which can modify the toxicant effect (see Kooijman and Metz, 1984; Jager et al., 2004). In DEB theory, food availability has predictable effects on the life-history characteristics, and this allows for simulations of the population growth rate under alternative conditions. Food limitation does not seem to affect the prediction based on short-term data; the population growth is simply shifted to lower values, in contrast to the synergistic effects of food limitation and toxicants, predicted for cadmium and triphenyltin in springtails (Jager et al., 2004). However, these chemicals had different energetic modes of action, which interact more directly with the food level. Clearly, the mode of action

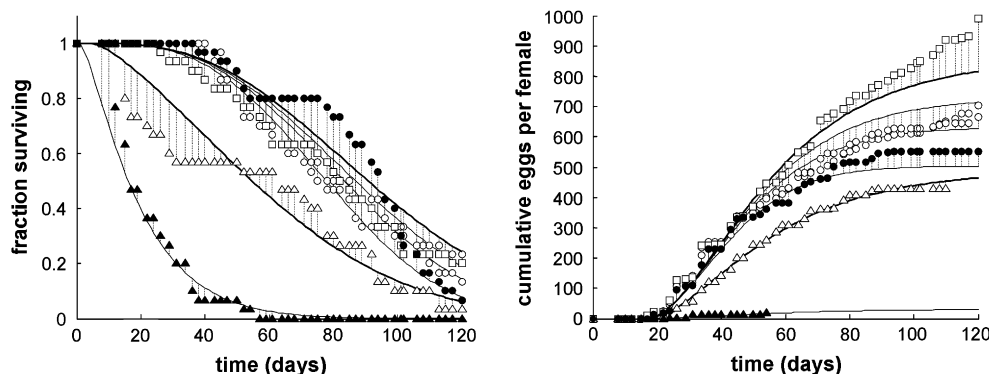


Fig. 3. Long-term survival and cumulative reproduction for *Folsomia candida* with separate models for high and low concentrations of chlorpyrifos: range 0–4.3 mg/kg_{food} (control response shown as open squares, highest concentration as filled circles, all intermediate concentrations as open circles) and range 9.3–20 (lowest exposure shown as open triangles, highest concentration as filled triangles). Details and parameter estimates in Table 1.

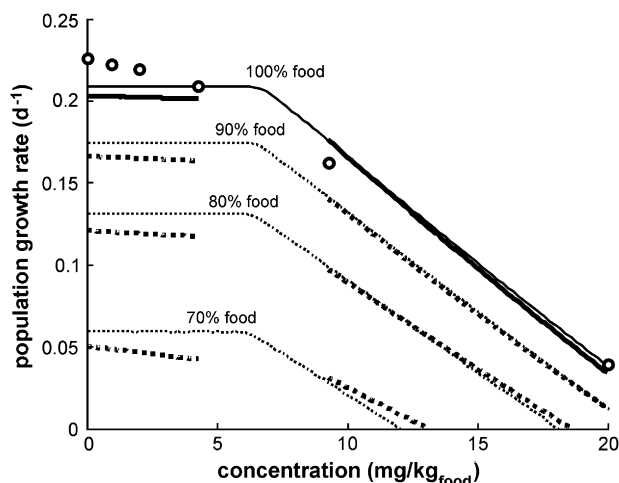


Fig. 4. Intrinsic rate of population increase as a function of chlorpyrifos concentration in food, and at different food levels (as percentage of the maximum ingestion rate), for *Folsomia candida*. Thin lines are for the short-term data (Fig. 2), thick lines are for the long-term data (Fig. 3, two modes of action). Dotted lines represent simulations at limiting food levels, symbols are directly calculated from the survival and reproduction data.

determines the response of the population under food limitation (see Kooijman and Metz, 1984). The ROS-based mode of action at low doses does seem to be slightly influenced by food level.

4. Conclusions

From the body size data (Fig. 1), it is clear that CPF in food does not affect growth, even at concentrations that almost completely inhibit reproduction. This contrasts results for cadmium and triphenyltin in the same species (Crommentuijn et al., 1997; Jager et al., 2004), as well as results for CPF in other species (the earthworm *A. caliginosa*, Booth and O'Halloran, 2001) where both growth and reproduction were affected. Nevertheless, our results imply that growth alone is not a suitable endpoint for risk assessment purposes; depending on the physiological mode of action (which is not only chemical but also species dependent), protecting against effects on growth may fail to protect against effects on reproduction, and thus against effects at the population level. This message is especially relevant because in many risk assessments and derivations of environmental quality criteria, no distinction is made between effects on growth or reproduction; if only one endpoint is available, that one is used. The current data set shows that no simple extrapolation between these endpoints exists.

The short-term data for survival and reproduction from the experiment are well described by the receptor model with a single mode of action for reproduction (direct hazard on egg production), revealing a prominent no-effect concentration. However, the exposure concentrations below this no-effect level do reveal dose-related negative effects on reproduction after a longer exposure time. Clearly, low doses of CPF in food reveal a different mode of action, only visible after

prolonged exposure, different from the rapid effects on survival and reproduction that are associated with AChE inhibition. The results suggest that these low-dose effects occur through the effects of CPF on the production of reactive oxygen species. Although there are indications that CPF can cause such effects, it remains speculative whether this is also happening in the springtails. Nevertheless, the current model provides excellent handles for dedicated experimental work (e.g. following AChE inhibition and oxidative damage in time), which can ultimately provide more insight into the mechanisms of OP toxicity and senescence, and the relations between these processes.

For risk assessment purposes, the intrinsic rate of population increase provides a better, more ecologically sound, representation of the response to toxicants than any single endpoint (Forbes and Calow, 1999). Because the early young are the most important from a population perspective, the peculiar low-dose effects, as observed in this study, are less relevant for risk assessment. Based on the current analysis, a 45-day life-cycle test would be sufficient for *F. candida* to provide an adequate estimate of the intrinsic rate of natural increase. However, care must be taken as the model predicts that these low-dose effects may be enhanced by an additional stress of food limitation.

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