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A mathematical model for the regulation of juvenile hormone titers

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Abstract

The titer of juvenile hormone (JH) is determined by three factors: its rate of synthesis, its rate of degradation, and the degree to which JH is protected from degradation by binding to a diversity of JH-binding proteins. All three of these factors vary throughout the life history of an insect and contribute to variation in the JH titer. The relative importance of each of these factors in determining variation in the JH titer is not known and can, presumably, differ in different life stages and different species. Here we develop a mathematical model for JH synthesis, degradation, and sequestration that allows us to describe quantitatively how each of these contribute to the titer of total JH and free JH in the hemolymph. Our model allows for a diversity of JH-binding proteins with different dissociation constants, and also for a number of different modes of degradation and inactivation. The model can be used to analyze whether data on synthesis and degradation are compatible with the observed titer data. We use the model to analyze two data sets, from *Manduca* and *Gryllus*, and show that in both cases, the known data on synthesis and degradation cannot account for the observed JH titers because the role of JH sequestration by binding proteins is greatly underestimated, and/or the *in vivo* rate of JH degradation is greatly overestimated. These analyses suggest that there is a critical need to develop a better understanding of the *in vivo* role of synthesis, sequestration and degradation in JH titer regulation.

Keywords: Juvenile hormone; Model; JH-binding proteins; JH titer regulation

1. Introduction

The juvenile hormones (JHs) of insects regulate an uncommonly broad diversity of developmental and physiological processes such as metamorphosis, reproduction, diapause, migration, seasonal polyphenism, and caste determination in social insects. Yet, in spite of more than half a century of studies on these ubiquitous insect hormones, their mode of action at the molecular level is still a mystery (Wheeler and Nijhout, 2003), and the mechanisms that control their level in the hemolymph and tissues remain incompletely understood.

It is generally agreed that the titer of JH is determined by a balance in the rate of its secretion and degradation, and by the degree to which JH is protected from degradation by binding to proteins in the hemolymph and in cells. There are several forms of JH in different species of insects, as well as a variety of binding proteins that differ in their abundance, in their localization, and in the strength with which they bind the different forms of JH (De Kort and Granger, 1996). An excellent and comprehensive recent review of the literature on the biology and biochemistry of JH is given by Goodman and Granger (2005).

Although secretion, degradation, and sequestration each must play a role in regulating the JH titer, it is not clear whether one of these factors is always more important than the others, or whether different factors control the JH titer at different times in an insect's life-cycle. One way to examine the relative roles of synthesis, degradation, and sequestration is by means of a quantitative mathematical description of the biochemical kinetics of these processes. A mathematical description makes explicit assumptions about how a process works, and is therefore useful for examining the consequences of those assumptions. The purpose of the present paper is to develop a quantitative theory for JH titers that can be used to study

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the significance of the various factors that are known, or suspected, to affect the levels of JH.

The kinetics of JH degradation and sequestration have been studied in many species of insects and are well enough understood for *Manduca* and *Gryllus* so that an explicit quantitative description is possible. During larval development of *Manduca sexta*, the JH titer fluctuates significantly, as does the rate of JH secretion, the activity of JH esterases (JHE), and the level of JH-binding proteins (Goodman, 1985; Hammock and Roe, 1985; Baker et al., 1987; Jesudason et al., 1990; Janzen et al., 1991; Hidayat and Goodman, 1994; Park et al., 1993). In adult *Gryllus firmus* there is a dramatic morph-specific daily cycle of JH titer that is accompanied by fluctuations in both JH synthesis and JHE activity (Zhao and Zera, 2004). We would like to find a method for calculating and predicting how fluctuating levels of JH synthesis, sequestration, and degradation control the JH titer and the half-life of JH in the insect.

2. Methods

2.1. Derivation of the kinetic equation for JH titers and half-life

The reaction system shown in Fig. 1 describes the synthesis and degradation of JH and its equilibrium binding to a protein that protects it from degradation. This system considers only one kind of JH, a single mode of JH degradation, and an indefinite number of JH-binding proteins with different affinities for JH. We note here that the binding protein term potentially includes other (yet unknown) sites like the fat body or cell membranes that bind JH and protect it from degradation. We develop a mathematical analysis of this system first, and then consider how it can be modified to deal with multiple modes of JH breakdown. The notational conventions we will use are JH_f for free JH (unbound to protein), BP for the free binding protein, and JHBP for the hormone–protein complex. We assume that the free JH is the active form of the hormone and that when JH is bound to a binding protein it is inactive. When JH titers in insect hemolymph are measured they refer to the total amount of JH_{tot} (free plus bound). Park et al. (1993) have shown that in *Manduca* only about 0.1% of the JH is free (i.e. about 99.9% of the JH is bound to protein), and the binding protein is in great excess so that 95% of the binding protein is free (i.e. has no JH bound). We assume that only free JH is subject to enzymatic degradation by JHE (Fig. 1). Although JH breakdown follows Michaelis–Menten kinetics, the K_m is much larger than the concentration of free JH (Hidayat and Goodman, 1994; Bonning et al., 1997), so the rate of JH breakdown is pseudo-first-order, and we assume it to depend on a single rate constant (k_2), which is approximately V_{max}/K_m. The derivation of k_2 is given in a separate section below.

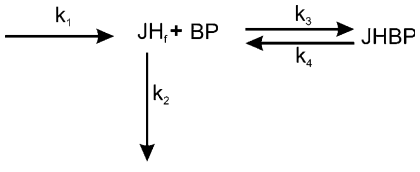

Fig. 1. Scheme of JH synthesis, degradation and sequestration. JH_f, free juvenile hormone; BP, free juvenile hormone-binding protein; JHBP, hormone–protein complex; k_1, rate of synthesis; k_2, rate of degradation; k_3, rate of binding to binding protein; k_4, rate of dissociation from binding protein. Although BP stands for any and all possible JH-binding proteins, this term also includes potential non-protein-binding sites such as plasma membranes and fat body inclusions.

2.2. Derivation of the kinetic equation for the JH titer

The concentration of each of the JH-binding proteins is generally much larger than the total concentration of JH, JH_{tot} (De Kort and Koopmanschap, 1989; Hidayat and Goodman, 1994; De Kort and Granger, 1996; Goodman and Granger, 2005), so that the concentration of free binding protein is not affected by JH_{tot}.

The concentration of free JH (JH_f) changes over time according to

$$\frac{\mathrm{d}}{\mathrm{d}t}(JH_f) = k_1 - k_2\,JH_f + \sum_{i=1}^{n}(k_4^i\,JHBP^i - k_3^i\,BP_{tot}^i\,JH_f), \tag{1}$$

where k_1 is the rate of synthesis, k_2 the degradation constant, so that $k_2 JH_f$ is the rate of degradation of free JH. Similarly, k_3^i and k_4^i are the association and dissociations constants, respectively, of JH with the ith binding protein, BP^i is the concentration of the ith binding protein, and $JHBP^i$ is the concentration of the ith hormone–protein complex. We note that it is possible for JH to bind and be sequestered by non-protein-binding sites. As long as this binding is reversible, it is accounted for in Eq. (1), and one can consider the ith binding protein in this equation (and all equations that follow) to represent a reversible non-protein sink for JH, with its own characteristic association and dissociation constants.

The concentration of each hormone–protein complex changes according to

$$\frac{\mathrm{d}}{\mathrm{d}t}(JHBP^i) = k_3^i\,BP_{tot}^i\,JH_f - k_4^i\,JHBP^i. \tag{2}$$

At equilibrium $\mathrm{d}/\mathrm{d}t(JH_f) = 0$, and $k_4\,JHBP^i = k_3\,BP^i\,JH_f$, thus, letting the dissociation equilibrium constants be $K_D^i = (k_4^i/k_3^i)$, we have

$$JH_f = \frac{k_1}{k_2} \text{ and } JHBP^i = \frac{BP_{tot}^i\,JH_f}{K_D}. \tag{3}$$

This means that at equilibrium, the concentration of free JH depends only on its rate of synthesis and degradation. Thus the binding proteins have no effect on the equilibrium

concentration of free JH, although they do affect how rapidly the equilibrium is reached.

The rate at which equilibrium is reached depends on the rate, k_3, at which JH dissociates from JHBP. For the *Manduca* JH-binding protein the half-time of dissociation is about 30 s (Park et al., 1993), so if this is the only protein present, the rate of decay of JH is determined almost entirely by the activity of JHE, because the rate of breakdown is substantially slower than the rate of dissociation. The dissociation rate constants are not known for any of the other JH-binding proteins. This is because the dissociation equilibrium constant of JH-binding proteins is typically determined by means of a Scatchard analysis (e.g. Tawfik et al., 2006), and this cannot tell us anything about the dissociation rate constant.

The total amount of JH (JH_{tot}) is the sum of the free JH and that bound to the various binding proteins, so

$$JH_{tot} = JH_f + \sum_{i=1}^{n} JHBP^i. \tag{4}$$

The concentration of JH_{tot} changes over time due to variation in synthesis and decay. Differentiating both sides of this equation, and using Eqs. (1) and (2), gives the following expression for the rate of change of JH_{tot}:

$$\frac{\mathrm{d}}{\mathrm{d}t}(JH_{tot}) = \frac{\mathrm{d}}{\mathrm{d}t}(JH_f) + \sum_{i=1}^{n} \frac{\mathrm{d}}{\mathrm{d}t}(JHBP^i) = k_1 - k_2 JH_f. \tag{5}$$

To solve Eq. (5), we must express $JH_f(t)$ in terms of $JH_{tot}(t)$. Assuming that the release of JH from the binding proteins is rapid relative to its rate of degradation, as the data from Park et al. (1993) indicate, we have (from Eqs. (3) and (4)),

$$\begin{aligned} JH_{tot}(t) &= JH_f(t) + \sum_{i=1}^{n} JHBP^i(t) \\ &= (1 + \textstyle\sum BP^i_{tot}/K^i_D)\, JH_f(t). \end{aligned}$$

Thus

$$JH_f(t) = \frac{JH_{tot}(t)}{1 + \sum BP^i_{tot}/K^i_D}. \tag{6}$$

Substituting Eq. (6) into Eq. (5), and solving, we obtain the JH concentration at time t:

$$JH_{tot}(t) = \frac{k_1 - (k_1 - \beta\, JH_{tot}(0))\mathrm{e}^{-\beta t}}{\beta}, \tag{7}$$

where

$$\beta = \frac{k_2}{1 + \sum BP^i_{tot}/K^i_D}. \tag{8}$$

Eq. (7) thus gives the concentration of JH at equilibrium as a function of its synthesis and decay rates, its sequestration by binding proteins, and its initial concentration $JH_{tot}(0)$.

2.3. Derivation of the kinetic equations for the half-life of JH

In order to calculate the half-life of JH we start with the system at equilibrium at time $t = 0$, so that by Eq. (3)

$$JH_f(0) = \frac{k_1}{k_2}, \tag{9}$$

and

$$k^i_3\, JH_f(0)\, BP^i_{tot} - k^i_4\, JHBP^i(0) = 0. \tag{10}$$

At equilibrium, and using Eq. (10), we find

$$\begin{aligned} JH_{tot}(0) &= JH_f(0) + \sum_{I=1}^{n} JHBP^i(0) = JH_f(0) \\ &\quad \times \left\{ 1 + \sum_{i=1}^{n} (BP^i_{tot}/K^i_D) \right\}. \end{aligned}$$

To compute the half-life of juvenile hormone we set k_1, the synthesis rate, to zero at $t = 0$, and use the differential Eq. (5) to calculate the time, $t_{1/2}$, so that $JH_{tot}(t_{1/2}) = JH_{tot}(0)/2$. In the absence of JH synthesis, the total concentration of JH is given by

$$JH_{tot}(t) = JH_{tot}(0)\mathrm{e}^{-\beta t}. \tag{11}$$

We can see that Eq. (11) is a special case of Eq. (7) for $k_1 = 0$.

Therefore $t_{1/2}$ satisfies

$$JH_{tot}(0)\mathrm{e}^{-\beta t_{1/2}} = JH_{tot}(0)/2,$$

hence solving for $t_{1/2}$ we obtain

$$t_{1/2} = \frac{\ln(2)}{k_2}\left(1 + \sum_{i=1}^{n} BP^i_{tot}/K^i_D\right) = \ln(2)/\beta. \tag{12}$$

The half-life of JH thus depends on the rate of degradation and the sum of the products of all binding proteins and their respective dissociation equilibrium constants. If there are no binding proteins, then $BP_i = 0$, and the half-life becomes

$$t_{1/2} = \frac{\ln(2)}{k_2},$$

which is the conventional equation for the half-life of a substance undergoing first-order decay.

If one wanted to consider the effect of additional degradative enzymes (e.g. JH epoxide hydrolase), then first-order rate constants can be calculated for those enzymes and simply added to the value of k_2 in all the preceding equations.

2.4. Apparent half-life of JH in the presence of JH synthesis

In the preceding derivation, we assumed that k_1 suddenly becomes zero. In reality, k_1 will decline over time and both the rate of decline and the new lower steady level will affect the half-life. Suppose that the systems starts at equilibrium $JH_{tot}(0) = k_1/\beta$, but the rate of JH synthesis (k_1) does not

drop to zero but to some intermediate value k_{next}. We can then calculate the half-life of JH as above, which yields the following equation:

$$t_{1/2} = \frac{\ln\left(k_1 - k_{next}/1/2\,k_1 - k_{next}\right)}{\beta}. \quad (13)$$

Again, we see that if $k_{next} = 0$, then $t_{1/2} = \ln(2)/\beta$, as before (see Eq. (12)). Furthermore, as k_{next} increases from zero toward $\frac{1}{2}k_1$, then $t_{1/2}$ will get longer and approaches infinity as k_{next} approaches $\frac{1}{2}k_1$. This finding implies that the observed, or apparent, half-life of JH is strongly influenced by the rate at which JH synthesis declines.

2.5. Derivation of the pseudo-first-order rate constant for JH-esterase activity

In order to apply the formulas derived above, it is necessary to estimate k_2, the first-order rate constant for JHE. JHE activity is usually measured under saturating JH conditions and recorded as nmol JH hydrolyzed per minute, per ml hemolymph (Hammock and Roe, 1985). This rate thus corresponds to the V_{max} of the enzyme in 1 ml of hemolymph. Breakdown by JHE is assumed to follow Michaelis–Menten kinetics.

In some insects, the concentration of JHE in the hemolymph is quite high and violates the assumption of the standard Michaelis–Menten equation that [substrate]-≫[enzyme]. When either the K_m or the enzyme concentration are much larger than the substrate concentration, the appropriate kinetic equation is $v = V_{max}[S]/(K_m + [E_t] + [S])$, where $[S]$ is $[JH_f]$ and $[E_t]$ is the total JHE concentration in the hemolymph (Cha, 1970; Brooks, 2004). If the K_m and E_t are much larger than $[S]$, then the rate of the reaction is linear in $[S]$ and is given by $v = V_{max}/(K_m + E_t)[S]$. The pseudo-first-order rate constant for JHE activity is therefore $k_2 = V_{max}/(K_m + E_t)$. Because the V_{max} is a function of total enzyme concentration: $V_{max} = E_t k_{cat}$, k_2 can also be written as $k_2 = V_{max}/(K_m + V_{max}/k_{cat})$. Thus the kinetics for JH degradation can be calculated from the value of the V_{max} if the K_m and either the E_t or k_{cat} for JHE are known.

The K_m's of JHEs from various insects range from 21 to 360 nM (De Kort and Granger, 1996; Bonning et al., 1997; Hinton and Hammock, 2003). The values for k_{cat} have been determined for the JHE of several species and range from 20 to 120 min^{-1} (Table 1). The k_{cat} for *Manduca* JHE is 104 min^{-1} (Bonning et al., 1997). The total JHE concentration has been much less studied. Abdel-Aal and Hammock (1985) give the value on the second day of the last larval instar of *Trichoplusia ni* as 1500 nM. The JHE concentration on day 3 of the 5th larval instar of *Manduca* can be estimated from the data of Venkatesh et al. (1990) as approximately 300 nM.

Thus in *Manduca*, where K_m of JHE is 30 nM (Bonning et al., 1997), a JHE "activity" of 1 $\text{nmol min}^{-1}\,\text{ml}^{-1}$ is equivalent to a V_{max} of 1000 nM min^{-1}, which corresponds to a pseudo-first-order rate constant of (1000 nM min^{-1})/(30 nM + 1000/104) = 25 min^{-1}. We will use this conversion factor throughout the analyses presented in this paper. In *Manduca*, JHE activity in the 5th larval instar varies from 0.3 to 72 $\text{nmol min}^{-1}\,\text{ml}^{-1}$ (Baker et al., 1987), which corresponds to a variation in k_2 of 7.5–1800 min^{-1}.

Table 1
Range of observed values for the various variables that affect the JH titer

Parameter	Observed range[a]	References[b]
k_1 (JH synthesis rate/pair CA) assuming 1 ml hemolymph volume	0–0.05 nmol min^{-1}	6,13,14,21,23,26
k_2 (JH breakdown rate)	0–50 nM min^{-1} 0–250 $\text{nmol min}^{-1}\,\text{ml}^{-1}$ 0–8250 min^{-1}	1,11,12,14,20
JH_{tot} (total [JH])	0–350 nM	1,5,13,26
JH_f (free [JH])	0.001–0.005 nM	5
JHE k_m	21–360 nM	2,16,19,26
JHE k_{cat}	20–120 min^{-1}	19,31,32,33
Manduca BP_{tot}	100–2000 nM	4
Manduca k_3 (association constant)	0.5–3.5 $\text{nM}^{-1}\,\text{min}^{-1}$	7
Manduca k_4 (dissociation constant)	0.5–3.5 min^{-1}	7
Manduca BP K_D	0.6–13.2 nM	5,7,8
JH-binding proteins K_D's	0.45–157	9,10,24,27
Lipophorins	1,000–70,000 nM	3,15,17,18,22
Lipophorins K_D's	20–33 nM	
Hexamerins	0–4 nM	8,10,30
Hexamerins K_D's	19–28 nM	
Locusta binding protein	11,000–26,000 nM	10,28,29
Locusta binding protein K_D	1.4–3.7 nM	

[a]Data from the literature were converted into units of nM, nmol and minutes.
[b](1) Baker et al. (1987), (2) De Kort and Granger (1996), (3) De Kort and Koopmanschap (1989), (4) (Goodman (1985); and personal communication), (5) Hidayat and Goodman (1994), (6) Janzen et al. (1991), (7) Park et al. (1993), (8) Tawfik et al. (2006), (9) Trowell (1992), (10) Braun and Wyatt (1996), (11) Sparks et al. (1979), (12) Sparks et al. (1983), (13) Treiblmayer et al. (2006), (14) Hoffmann et al. (1995), (15) Tsuchida and Wells (1988), (16) Campbell et al. (1998), (17) De Kort and Koopmanschap (1987), (18) Trowell et al. (1994), (19) Engelmann and Mala (2000), (20) Jesudason et al. (1990), (21) Niimi and Sakurai (1997), (22) King and Tobe (1993), (23) Holbrook et al. (1997), (24) Touhara et al. (1993), (25) Bloch et al. (2000), (26) Zhao and Zera (2004), (27) Vermunt et al. (2001); (28) Braun et al. (1995); (29) Koopmanschap and De Kort (1988); (30) Ismail and Gillott (1995), (31) Vermunt et al. (1997), (32) Abdel-Aal and Hammock (1985), (33) Abdel-Aal and Hammock (1988).

2.6. Variables

Table 1 gives the ranges of values of the variables involved in hemolymph JH titer regulation. A cautionary note is in order here. The measurement and interpretation of JH titers has been fraught with uncertainty and controversy. Besides the well-known fact that the extraction and purification of JH can be variably inefficient

because of losses due to binding to glassware and the extraction apparatus, different techniques for quantification of JH give different results. The antibodies used for RIA studies bind different types of JH with significantly different affinities, GC/MS methods can only quantify the kinds of JH for which the system has been calibrated, and bioassays can quantify total JH activity but cannot identify what molecules are involved. Hence, the data in Table 1 refer to JH generically, and for publications that reported the titer of more than one kind of JH, the total JH concentration is simply assumed to be the sum of all identified kinds of JH. The measurement of JHE activity is likewise variable and inconsistent. For instance, the peak level of JHE activity during the last larval instar of *Manduca* has been reported as $10\,\mathrm{nmol\,min^{-1}\,ml^{-1}}$ (Jones et al., 1982), $22\,\mathrm{nmol\,min^{-1}\,ml^{-1}}$ (Venkatesh and Roe, 1988), $45\,\mathrm{nmol\,min^{-1}\,ml^{-1}}$ (Weirich et al., 1973; Vince and Gilbert, 1976), $60\,\mathrm{nmol\,min^{-1}\,ml^{-1}}$ (Browder et al., 2001), $70\,\mathrm{nmol\,min^{-1}\,ml^{-1}}$ (Baker et al., 1987), and $100\,\mathrm{nmol\,min^{-1}\,ml^{-1}}$ (Sparks et al., 1983). These reports span an order of magnitude and it is unclear whether this is due to differences in strains used, or differences in non-specific JH esterases, or technique. Almost all the data on rates of JH synthesis are obtained from corpora allata (CA) cultured *in vitro*, which are not under normal regulation and may therefore not accurately reflect the activity *in situ*. Importantly, the model for JH titer regulation developed above can be used as a tool to test whether experimentally obtained figures for the variables of JH synthesis, sequestration and decay are in a reasonable range, and whether they make sense in the context of other available data. The model can also be used to determine whether available data are (or, as we will see, are not) sufficient to account for the observed titers and half-life of JH. We will give several examples in the analyses below.

3. Results and analysis

We have developed a set of equations that describe the time-varying titer of JH, the steady-state concentration of bound and free JH, and the half-life of JH, as functions of the rate constants of synthesis, degradation, association, dissociation, and the concentration of the binding protein.

The equations apply to the interaction of a single species of JH with an array of binding proteins with different concentrations and dissociation constants. In reality, most insects express several different forms of JH, each with different synthesis, degradation, and binding kinetics. Because the binding proteins are in excess, the different species of JH do not compete for binding, and thus their kinetics of synthesis, binding, and degradation are independent of each other, and each will obey the equations derived above.

We see that the steady-state concentration of JH depends only on the rate of synthesis and breakdown (Eq. (3)), and does not depend on the amount of JH-binding protein present (although the amount of binding protein and JH_{tot} will affect how rapidly the steady state is achieved). We also see that the half-life of JH depends on the four parameters and is a linear function of the amount of binding protein (Eqs. (12) and (13)).

Thus to predict the titers of free and bound JH, and its half-life, it is necessary to obtain values for its rate of synthesis and degradation, of the dissociation equilibrium constant with each binding protein, and of the concentrations of those binding proteins. Unfortunately, several of these are notoriously difficult to obtain, in part because of the difficulty of controlling and accounting for non-specific binding of JH to other proteins and to the walls of the reaction vessels, and in part due to the inability to accurately replicate *in vitro* the conditions that obtain *in vivo*. The uncertainty in, or ignorance of, any of these factors, limit our ability to accurately measure the kinetics of JH titers empirically.

The important feature of the models is that they relate the contributions of these factors, and they make the nature of their interactions explicit. This is sufficient for us to draw conclusions about where and when each of these regulators of JH are likely to matter the most, and also about the relative importance of each. Even in the complete absence of experimental data on the kinetics of JH, the quantitative theory we developed above can be used to examine the relative roles of all the variables in determining the kinetics of JH titers. In addition, where data are available, the theory can be used to calculate whether they are consistent with other results obtained for that system. For example, one can use Eq. (12) to determine if the measured half-life of JH in one experiment is consistent with independent measurements of its interactions with the binding protein. We show examples of this below.

To start, it is useful to do an analysis of Eq. (12), which gives the half-life of JH in the absence of JH synthesis. Suppose that there is a single binding protein with a K_D of 10 nM, that the first-order rate of JH degradation is $1\,\mathrm{min^{-1}}$, and that the concentration of the binding protein is 1000 nM, then the half-life of JH is $0.693(1+1000/10) =$ about 70 min. The equation shows that doubling the concentration of binding protein (all other things being equal) will double the half-life of JH, and doubling the rate of JH degradation (e.g. by doubling the concentration or activity of JHE) will halve the half-life of JH.

Since there is surely a diversity of JH-binding sites in the hemolymph and in tissues, it is useful to develop a general scheme that would allow us to establish the conditions required to obtain a given half-life of JH. Eq. (12) allows us to plot graphically the relationship between the half-life of JH, the activity of JHE, and the "activity" of the binding proteins. We define a new variable, α, to represent sum of the ratios of binding protein concentration and their respective K_D's, so we can plot the half-life of JH as a function of JHE activity (k_2) and the sum of all the binding sites that play a role in a given system (Fig. 2).

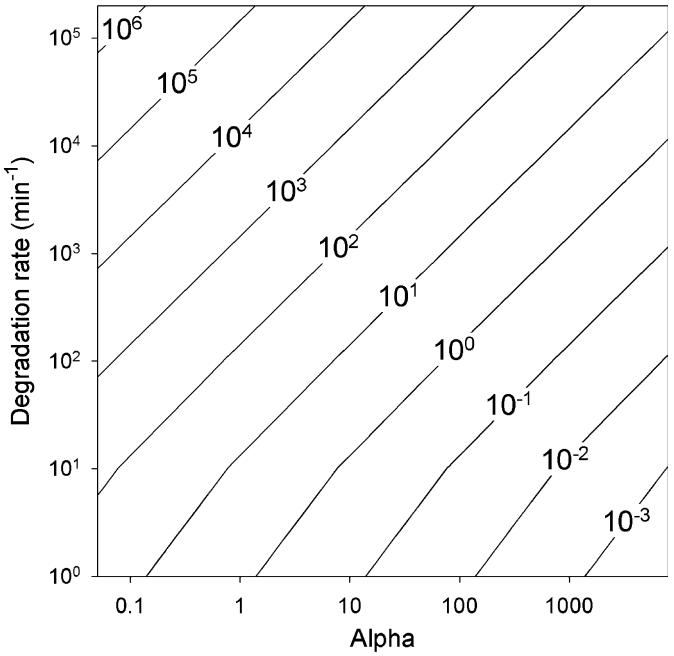

Fig. 2. Half-life of JH (in min) as a function of JH decay rate and JH protection by binding proteins. Alpha is the sum of the ratios of binding protein concentration and their respective K_D's.

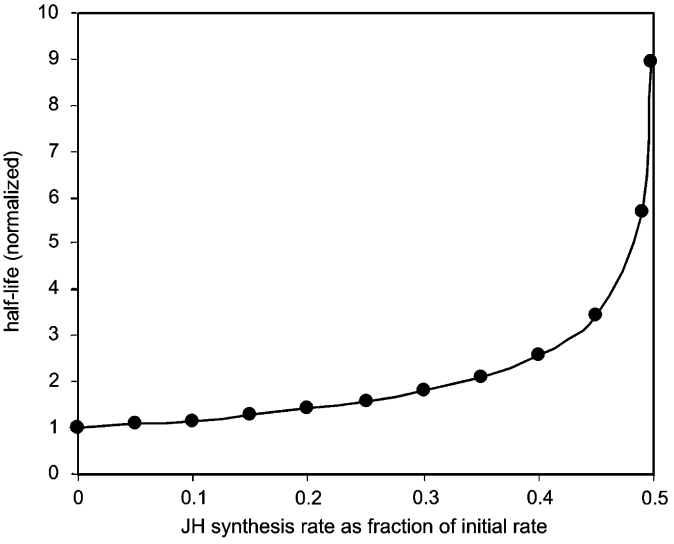

Fig. 3. Apparent half-life of JH with declining JH synthesis, based on Eq. (13). The X-axis is k_{next} of Eq. (13). The graph is normalized to 1, so that if JH synthesis declines to zero the half-life is given by Eq. (12), and if the rate of synthesis declines to 40% of its initial rate the half-life is approximately 2.6 times the value given in Eq. (12). If the rate of JH synthesis declines to a level higher than 50% of its initial value the half-life is effectively infinite and cannot be calculated.

If JH synthesis declines gradually, the apparent half-life of JH will be longer and is given by Eq. (13). Fig. 3 shows the relationship between the half-life of JH and the level, k_{next}, to which JH synthesis declines. If the rate of synthesis does not decline to less that half its initial rate, the apparent half-life of JH will be infinite (and thus cannot be measured).

3.1. An example from Manduca

Now we take a real example from *Manduca sexta*, where the concentrations and binding properties of the JH-binding protein are well known (Goodman, 1985; Park et al., 1993; Hidayat and Goodman, 1994). The concentration of this binding protein fluctuates during larval life from 100 to 2000 nM. This protein has a K_D that varies from 0.6 to 1.9 nM, depending on the type of JH and on the conditions under which it is measured (Park et al., 1993). We will take the value as 1.5 nM for our example. The JHE activity of *Manduca* has also been well studied and has been shown to fluctuate widely, depending on the developmental stage, and ranges from 0.3 nmol min^{-1} ml^{-1} to brief peaks as high as 72 nmol min^{-1} ml^{-1} as noted above, corresponding to a range of pseudo-first-order decay constant of 7.5–1800 min^{-1}.

Taking the concentration of the JH-binding protein at the end of the 5th instar to be 600 nM, and the JHE breakdown rate to be at its peak of about 1800 min^{-1}, Eq. (12) predicts the half-life of JH to be $0.693/1800(1 + 600/1.5) = 0.15$ min.

This value is substantially shorter than the estimated half-life of about 25 min (Nijhout, 1975). We can obtain an independent approximation of the JH half-life during the 5th instar of *Manduca* from the data of Baker et al. (1987). These authors obtained JH concentrations at 12 h intervals, and the rate of decline during those intervals can be used to calculate a lower limit to the half-life of JH during those intervals (assuming the rate of decay is constant during each 12 h interval). During the first three 12 h intervals of the last larval instar, total [JH] declines gradually from 10.4 to 1.65 to 0.45 and finally to 0.035 nM. This corresponds to half-lives of 4.8, 6.0, and 3.4 h during each of these intervals. Clearly, these half-lives are substantially longer than 0.15 min, indicating that something is missing from the assumptions made in the above calculation.

To obtain the observed half-life either the activity of JHE must be much lower than reported by all authors who have measured JHE activity by a factor of 160 (for a half-life of 25 min) to 1300 (for a half-life of 4 h), which seems unlikely. Alternatively, the concentration of the hemolymph-binding sites must be much higher than reported (also unlikely), or there are additional binding sites that protect JH from degradation, or there is some JH synthesis during the period that JH titers decline. We will examine each of the latter two alternatives in turn.

Lipophorins are a class of lipid storage and JH-binding proteins that occur at high concentrations in the hemolymph. In 5th instar larvae of *Manduca*, the lipophorin concentration is about 3 mg ml^{-1} (Tsuchida and Wells, 1988). Assuming a molecular weight of about 250 kDa (Prasad et al., 1986; Canavoso et al., 2001), this gives a concentration of approximately 12,000 nM. The *Manduca* lipophorin binds JH with a K_D of 25 nM.

In the summation term of Eq. (12), the contribution of the *Manduca* JH-binding protein (at the time point used

above) is $600/1.5 = 400$, and that of the lipophorin is $12{,}000/25 = 480$. Thus we could say that the lipophorins account for roughly 50% of the JH-protective capacity.

To obtain a half-life of 60 min would require $0.693/3000(1+x) = 60$ min, where x is the summation term in Eq. (12), so $x = 60/0.00023 =$ about 261,000. The sum of the BP/K_D ratios of the JH-binding protein and of lipophorin is only 880, so an additional 260,000 must be made up by other JH-binding sites. Thus the calculations show that the combined JHBP and lipophorin account for only a very small fraction of the JH-protective activity that regulates the JH titer in 5th instar larvae of *Manduca*. One possibility is that plasma membranes, the fat body, and other issues serve as large sinks or reservoirs for JH. If this is correct, then the prediction of the model is that for this entire reservoir the sum of the molarity of JH-binding sites divided by their respective K_D's, must be in the neighborhood of 260,000.

3.2. An example from Gryllus

Zhao and Zera (2004) provide a unique and interesting data set on the simultaneous rate of JH synthesis, JHE activity, and JH titer in adult *Gryllus firmus*. In the long-wing morph, there is a circadian rhythm of JH titer, which appears to be driven by corresponding circadian variations in JH synthesis and JHE activity. In Fig. 4 we digitized the data from Fig. 3 of Zhao and Zera (2004) for the period between day 5.4 and day 6.4 and rescaled the *Y*-axis to correspond to the units used in our model, so that time is counted in minutes and concentration in nM. The *X*-axis is scaled in 0.1 days as in Zhao and Zera (2004). For JH synthesis, we assumed that the CA secrete into a volume of 100 µl, so the rate of synthesis is given as nM min^{-1}. This transformation is required to keep the units consistent with those used in the model. If the hemolymph volume is smaller, say 10 µl, then the predicted concentrations would, of course, be 10-fold higher than shown in Fig. 4A.

If we assume that each data point in Fig. 4A represents an equilibrium between synthesis and breakdown, then we could apply Eq. (3) to the data in Fig. 4B and C to calculate the predicted JH titer (i.e. $k_1(t)/k_2(t)$). The dashed line in Fig. 4A shows this prediction. Clearly, this assumption is incorrect. We simplified the data profiles of JH synthesis and degradation as shown in Fig. 5B and C. We assumed that the JH titers were measured with great accuracy and we adjusted only the heights of the profiles of JH synthesis rate (Fig. 5B), and breakdown rate (Fig. 5C), not their duration or relative timing. We then solved Eqs. (5) and (6), putting in the time-varying $k_1(t)$ and $k_2(t)$. The results of these simulations are shown in Fig. 5A. For JH breakdown, we took into account the interaction between JHE activity and protection of JH by binding proteins, thus the *Y*-axis of Fig. 5C is

$$\frac{k_2}{1+\sum BP^i_{tot}/K^i_D}, \tag{14}$$

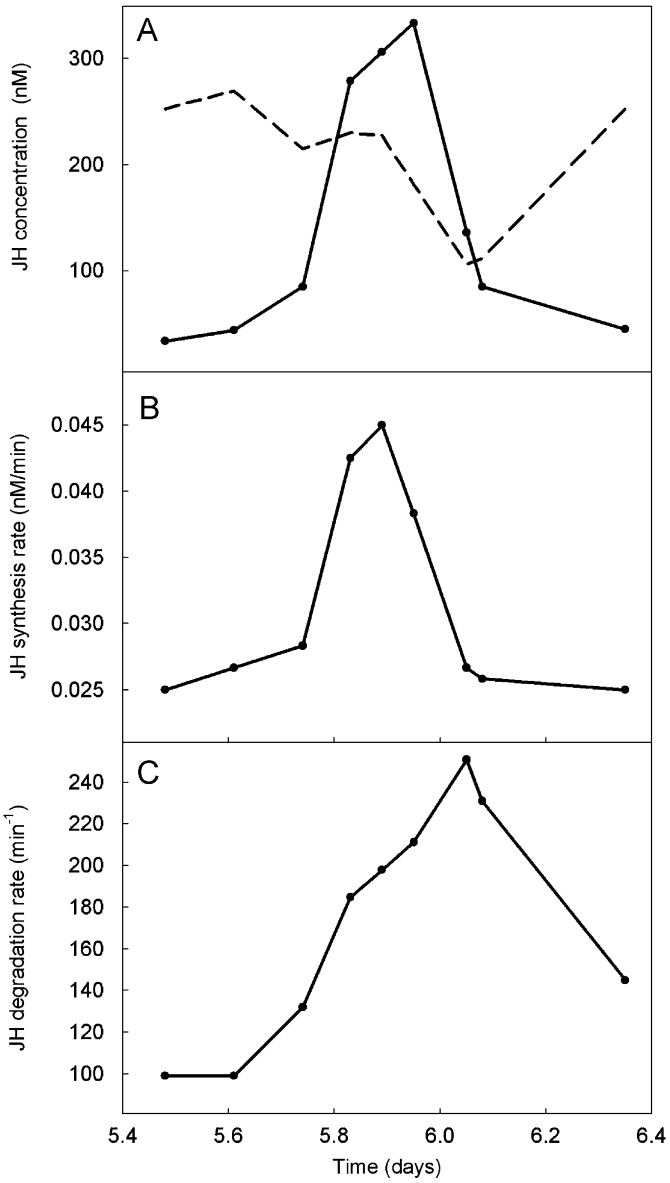

Fig. 4. Data on JH titers (A), *in vitro* synthesis (B) and JHE activity (C) in *Gryllus*. Redrawn from Zhao and Zera (2004). Mean values adjusted to common units. Dashed curve in panel (A) is the predicted concentration of free JH from the synthesis and degradation data in panels (B) and (C), using Eq. (3), assuming rapid-equilibrium conditions as discussed in the text. To obtain the rage of values observed for the JH titer, the predicted steady-state concentration of free JH needs to be multiplied by a scaling factor of 1,000,000 indicating that the rapid-equilibrium assumption is not applicable, presumably due to the effects of protective binding proteins.

that is, the rate of breakdown by JHE divided by the sum of the concentrations of binding proteins and their dissociation equilibrium constants. It is clear that the model can produce an accurate representation of the observed JH titer, but not with the observed data on JH synthesis and JHE activity alone. The analysis suggests that the rate of JH synthesis is greatly underestimated. This is also clear from the data themselves, which show that the JH titer varies by a factor of about 6 during the daily cycle (Fig. 4), whereas JH secretion (by cultured CA–corpora

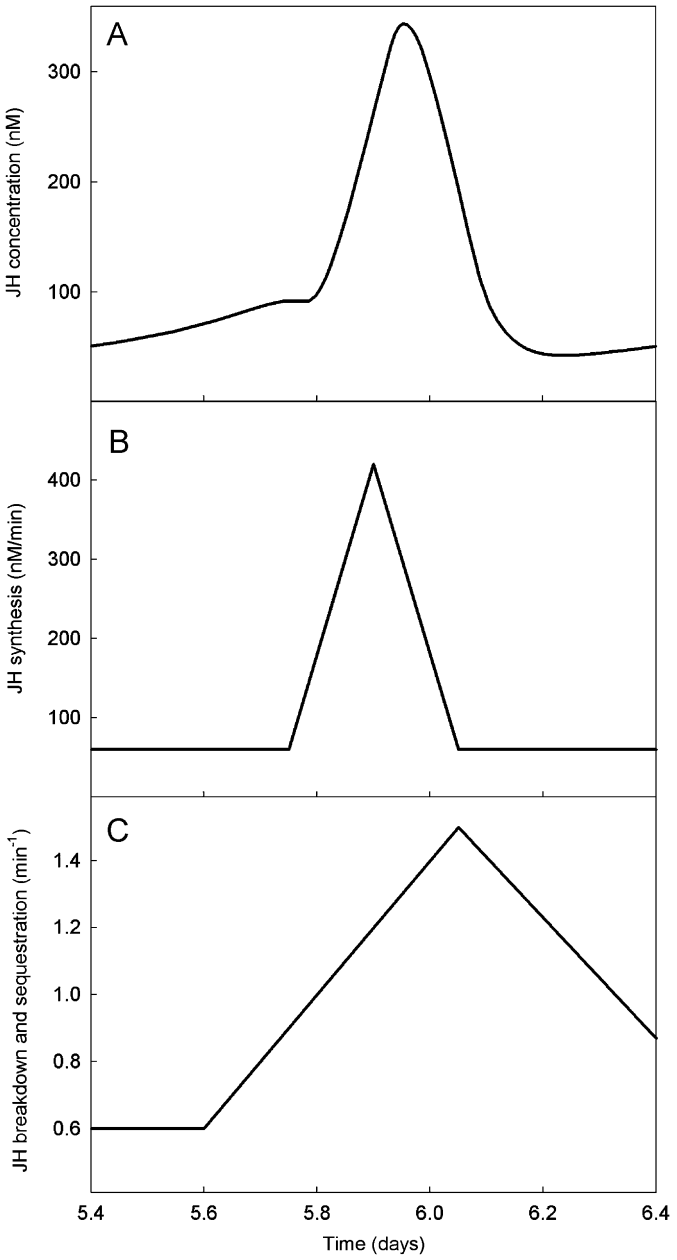

Fig. 5. Simulation of the data in Fig. 4, using Eqs. (5) and (6). Predicted JH titer profile (A) from the pattern of JH synthesis (B) and a combination of degradation and sequestration (C).

cardiaca (CC) complexes) shows only a two-fold variation in synthesis rates during a rising phase of JHE activity. It is difficult to see how a two-fold change in synthesis rate can give rise to a six-fold change in concentration (all other things being equal). Note that the *Y*-axis in Fig. 5C contains contributions from both JH degradation (numerator of Eq. (14)) and JH sequestration (denominator). The values for the *Y*-axis in Fig. 5C differ from those in Fig. 4C by a factor of 167. This implies that in deriving the *Y*-axis in Fig. 4C (using the equation $V_{max}/(K_m + V_{max}/k_{cat})$), either the value of k_2 was overestimated by a factor of 167, or the contribution of JH binding and sequestration was underestimated by a factor of 167 (it was assumed to be 0; see Eq. (14)), or a combination of both. Only experimental data will be able to determine how the relative effects of degradation and sequestration are partitioned.

4. Conclusions

We have provided a quantitative theory of JH titers that takes into account JH synthesis, JH degradation, and the sequestration and protection of JH by binding to a variety of specific and non-specific binding proteins. A study of the causal analysis of the total JH titer or the free JH titer needs to take all these factors into account. Our model is a mathematical description of the common assumptions made by investigators working on the dynamics of JH titer regulation.

The theory we have developed allows one to predict the ranges of values of unknown variables based on partial information, for instance, whether data on JH synthesis and degradation and sequestration are consistent with the observed JH titers. Take, for instance, Eq. (12), which describes the half-life of JH. If both the half-life and the decay rate of JH are known, this equation predicts the total JH-binding capacity of sequestering proteins. If the properties of one of those proteins are known, then the equation predicts whether or not one needs to search for additional binding sites, and if one is confident that all binding sites have been accounted for (and the left and right sides of the equation still do not balance), then one would conclude that maybe some remnant JH synthesis plays a role and one should use Eq. (13) instead.

Analyses with the model suggest that it cannot be safely assumed that variation in JHE completely accounts for variation in JH titers. The role of JH synthesis can be substantial, but it appears that this rate is underestimated in studies that use cultured CA. It is also clear that the role of JH-binding proteins is greatly underestimated. In *Manduca*, the known hemolymph-borne JH-binding proteins alone cannot account for the observed sequestration and protection of JH, suggesting that a tissue like the fat body must play a major role. And in *Gryllus*, the time-varying profiles of JH cannot be obtained from time-varying synthesis and degradation alone, but must also take into account the binding proteins. Our model shows that even the extremely high BP concentrations (with high-affinity binding sites) that have been measured still do not leave all JH bound, and we show clearly that the behavior of these binding proteins can significantly affect the speed of temporal fluctuations in available JH. Realistic appraisals of biologically meaningful variation in JH levels must therefore include information about circulating levels (and identities) of the various JH-binding proteins, as well as the non-protein sinks for JH. Finally, it is possible that JHE activities measured *in vitro* are a poor reflection of JHE activities *in vivo*. There is a reciprocal relationship between JHE activity and sequestration by binding proteins in

establishing the JH titer dynamics. This is why we use the parameter β in Eqs. (7) and (13) and in defining the Y-axis in Fig. 5C. This parameter allows one to ascribe the mismatch of experimental data to a combination of effects of breakdown and sequestration.

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