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得獎獎項　大會獎：三等獎

荷蘭 INESPO 正選代表：2014 年荷蘭國際環境及永續發展競賽

就讀學校　國立科學工業園區實驗高級中學

指導教師　孫以瀚、葉柏安

作者姓名　陳昌逸、陳慶豐

關鍵字　<u>NPF、GAL4-UAS</u>

作者簡介

我是陳昌逸，就讀新竹實驗高中數理班三年級，並於一年級開始參加中研院人才培育計畫。我對科學產生興趣，來自幼兒時對 Discovery 和 Animal Planet 的著迷，科學對我來說其實並不是一個「讀書把考試考好」的學科而已，而是與我們本身及周遭事物息息相關的學問。經過這次實驗的歷練，我更是深刻了解科學尚未探知的事物，永遠比已知的多許多。

我是陳慶豐，現在就讀新竹實驗高中數理班三年級。一開始會打算做關於神經的題目，是抱著一份對“心理學”的熱愛。原本以為神經科學與心理學是密不可分的，沒想到實際進行實驗設計、操作、與觀察，才發現微觀和巨觀尺度的距離，其實相差了的是十萬八千里，就彷彿中西醫的差距：一個是從分子化學出發，一個是做行為實驗的歸納。但神經科學也有它的趣味在其中，科學研究真的是有趣的令人難以自拔!

中文摘要

研究指出，公果蠅在求偶的過程中若被母果蠅拒絕，腦中的神經傳導蛋白NPF(neuropeptide F)會隨之下降，並提升其對酒精食物的攝取量。

我們的研究結果顯示求偶被拒的公果蠅是在「攝食酒精」後才對酒精成癮，而非在求偶被拒之後馬上去攝食酒精。而且,「求偶被拒」的公果蠅除了飲酒行為，也會提升其再求偶慾望。另外，我們利用 Gal4-UAS 系統調控了公果蠅腦中 NPF 神經的活性，發現公果蠅的 NPF 神經活性被抑制時，會出現與求偶被拒的公果蠅類似的行為。

最後，我們使用了 NPF 的螢光蛋白的抗體來標定 NPF 神經活性被激發以及抑制時腦中 NPF 的含量變化。透過這個結果，我們可以確定腦中 NPF 量的變化可以控制公果蠅對酒精的趨性、求偶慾望以及對酒精的攝食量。

Abstract

A study published on Science showed that male fruit flies, which are rejected by female flies during courting, tend to take more alcoholic food. The same study also demonstrates the concurrent decrease of a neurotransmitter Neuropeptide F(NPF) in the brains of rejected male flies. Therefore, this study is to explore the correlation between the change of ethanol preference and the decrease of NPF in male fruit flies' brain after being rejected by female flies during courting.

Our results showed that the ethanol food consumption response after rejection can be separated into two stages. Right after the four-day rejection, the male flies actually avoid ethanol. However, after ethanol consumption, their ethanol avoidance becomes reversed to ethanol preference that is stronger than normal. Moreover, the rejection also caused a number of ‘’emotion-like” behavior changes, including courting motivation and ether consumption.

And the results showed that flies with lower NPF amount in their brains may change their behaviors similar to those caused by courtship rejection. Thus our results demonstrated that rejection cause the decrease of NPF, which in turn cause a number of behavior changes. Therefore, we can argue that fruit flies' reactions are not only controlled by ‘’simple reflex behaviors’’ but induced by emotion-like behaviors. The results can potentially help clarify the function of the NPY (NPF's homolog in human)..

壹、　前言

一、研究動機

2012 年，在國際期刊 Science 刊出的一篇研究指出，果蠅在求偶失敗的時候，會比一般果蠅更偏好於食用含有酒精的食物。這令我們十分好奇，昆蟲真的會有人們「藉酒澆愁」的行為嗎?

酒精在自然界中扮演的角色可是相當的複雜，它是許多生物的食物，卻也是一種無專一性的神經鎮定劑。研究中指出公果蠅在被拒絕後，偏好食用酒精的同時，腦中一種稱為 NPF 的神經傳導物也會有下降的現象，然而卻沒有明確的指出 NPF 量的變化及公果蠅食用酒精行為的關連性。因此我們想藉由果蠅行為上的觀察，並利用 Gal4-UAS 技術直接改變腦中 NPF 的量，以找出公果蠅被拒絕、NPF 的增減與果蠅「酗酒」之間的關聯性，最後再利用文獻探討找出情緒(emotion)的嚴謹定義，討論公果蠅被拒絕後的行為改變，亦即酒精成癮是否是因為酒精讓其情緒狀態(emotional state)產生轉變。

二、背景介紹

(一)果蠅(***Drosophila melanogaster***)

果蠅(*Drosophila melanogaster*)是種適合進行生物實驗的模式生物，其體積小、生命週期短、擁有許多基因工具、飼養成本低廉，且染色體只有四對，相較於人類的二十三對要少許多，研究起來也比較單純。除此之外，果蠅在聽覺、味覺、視覺、痛覺、嗅覺、學習及記憶等認知行為與人類也有許多相似之處，根據統計人類有百分之七十的神經疾病在果蠅身上都找的到相同或類似的症狀。藉由研究果蠅，將可以對腦神經運作的了解有很大的幫助。

Fig.1 公果蠅(下)正在向母果蠅求愛

果蠅的求偶行為(courtship behavior)複雜，其中包括了:定向、輕觸、追逐、振翅、舔舐、嘗試交配、交配。由於求偶行為複雜，其中參與的神經路徑也很複雜。而若母果蠅已經和其他的公果蠅交配過了正在產卵，這時無論公果蠅如何努力都不會得到對方的回應，這種狀況就是我們所謂的「被拒絕」。有趣的是， (Ophir et al.,2012)指出果蠅在被拒絕後，竟會增加酒精的攝食量，引起我們的好奇，想理解其增加酒精攝取量的原因。

(二)Gal4-UAS system

我們利用 Gal4-UAS 系統將腦中 NPF 的量降低。這個系統的原理，是利用基因轉殖技術，使即將交配的雌雄果蠅，一方帶有啟動子(endogenous promoter/enhancer) 和 GAL4 基因，另一方帶有結合子 UAS (GAL4 binding site)和欲研究的基因。在兩者交配後，其子代中 GAL4 會和 UAS 結合。Gal4 有如導航，將如同鑰匙般的 GAL4 和 UAS 結合，便可觸發下游欲研究的基因表現。我們利用只表現於 NPF 神經細胞的 NPF-Gal4，在表現於 NPF 的神經細胞中，驅動 UAS-kir2.1 的表現。Kir2.1 可增加神經細胞的鉀離子通道反應率，使神經細胞無法被去極化，因此神經活性被抑制 (Baines et al., 2001;Paradis et al., 2001)。我們也利用 NPF-Gal4 去驅動 UAS-dTrpA1 在 NPF 神經細胞中的表現。TrpA1 會抑制鉀離子通道的反應率，使神經細胞容易被去極化，其神經活性被增強 (Rosenzweig et al., 2005;Rosenzweig et al., 2008)

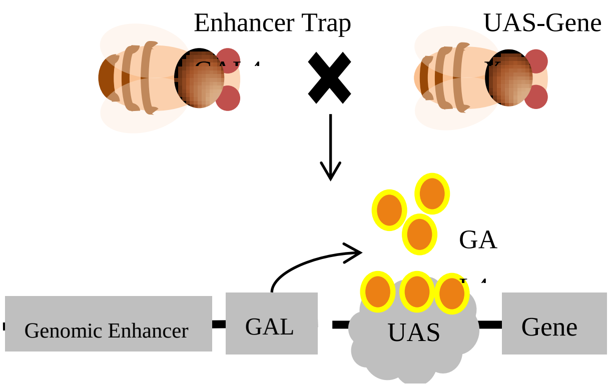


Fig.2 UAS-GAL4 的使用模式

(三)神經胜肽 F(Neuropeptide F)

NPF 這種神經傳導物質，是哺乳類神經傳導物 NPY 的同源體(homolog)。它們兩個有相當多共通的功能，例如影響生物體食物的選擇、誘發肥胖以及控制該生物之活動力..等等。而 NPY 也被發現會在哺乳類的腦中產生焦慮、害怕、緊張…等負面情緒(Wu et al.,2003)，不禁令我們感到好奇，NPF 是否與 NPY 一般，也會在公果蠅腦中產生類似的情緒?

(四)情緒(emotion)

情緒這個概念通常被我們運用在擁有高等神經系統的脊椎動物身上，因為我們比較能夠用他們的神情、叫聲、需求..等證據中推想他們正處於什麼情緒狀態(Dawkins,2008)，造成我們比較能夠感同身受並理解他們的遭遇。而果蠅這類行為簡單的模式生物，有沒有可能發展出由情緒這個動機，引起我們的好奇。

2009 年時，加州理工學院的 David Anderson 教授所帶領的研究團隊從果蠅身上發現了一種由多巴胺控制的原始「類情緒驅動行為」(emotion-like behavior)，並定義出由情緒所驅動的行為需要符合的三樣條件 1. Persistent 2. Graduation od intensity 3. Valence，開啟了後人從果蠅身上研究情緒的里程碑(Anderson,2009)。這類研究是重要的，因為現今大多數人類擁有的神經疾病皆可以從果蠅身上發現並尋找方式解決，很多這一類型的行為異常被發現為一些現代精神疾病的基礎。

貳、　研究方法與過程

一、研究設備與器材

(一) **實驗材料**：果蠅(*Drosophila melanogaster*）、飽和葡萄糖溶液、純酒精、乙醚、飼料

(二) **實驗器材:** 解剖顯微鏡、毛細管(75mmx1.15mm)、電子秤、玻璃管、玻璃瓶、共軛焦螢光顯微鏡，蓋玻片、觀察操作台、T 型三向管，細鑷子

Fig.3 果蠅管飼養盤 10*10

Fig.4 玻璃瓶中飼養的果蠅

Fig.5 觀察操作台、解剖顯微鏡

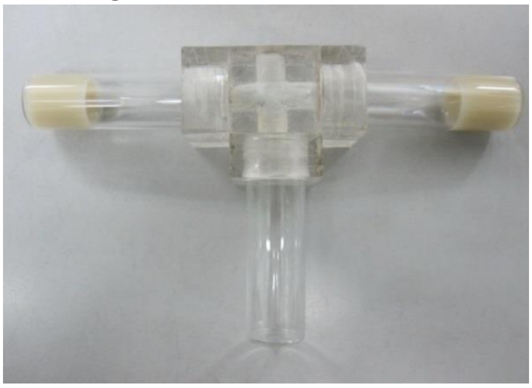


Fig.6 自製的 T 型迷宮

Fig.7 共軛焦螢光顯微鏡

Fig.8 用蓋玻片製作之標本、準備大腦用的溶液

二、研究過程與方法

(一) 實驗項目

階段一：文獻實驗的重複以及前置實驗

1. 用 15%的酒精來進行實驗恰當嗎?

2. 乙醚食物的濃度大約多少才恰當?

3. 公果蠅在求愛被拒絕之後，是否真的會增加酒精的攝食量?

4. 酒醉的公果蠅要花多少時間回復爬行速度?

階段二:酒精如何在被拒絕的公果蠅腦中產生作用？

5. 求偶被拒真的會直接增加對酒精的趨向嗎?

6. 求偶被拒後的大腦的反應是不是“降低對酒精趨向”?

7. 求偶被拒與的公果蠅是否會增加求偶慾望?

8. 求偶被拒的公果蠅使否會增加對乙醚的攝食量?

階段三：神經傳導物質 NPF 量的變化對公果蠅行為的影響為何？

9. NPF 神經的活性是否會影響公果蠅對酒精的攝食量?

10. NPF 神經的活性與是否會影響公果蠅對酒精的趨向?

11. NPF 神經的活性是否會影響公果蠅的求偶慾望?

12. NPF 在腦中作用位置觀察

13. NPF 神經的活性改變是否真的可以改變 NPF 在腦中的量?

階段四:探討求偶被拒絕的公果蠅的行為改變是否符合「類-情緒行為」的定義

14. 求偶被拒後行為改變是否持續?

15. 不同拒絕程度是否造成公果蠅行為改變程度上的差異?

攝取量實驗以及趨向實驗:

攝取量實驗是在飼養的流程結束後，將公果蠅放入空管記錄其在 12hr 內對兩種食物的攝食量。而趨向實驗則是在飼養的流程結束後，將其放入三向道中三分鐘，計算選擇 1. 含酒精食物以及 2. 正常食物 的比例。

兩種實驗最大的差異在於:

公果蠅在 12hr 的期間內會發生很多很多事，所以其對酒精食物攝取量的比例是所有發生的事情加總的結果。但是趨向實驗則是盡量把所有可能發生的事情的步驟拆開，觀察他們在不同情形下三分鐘內對酒精食物的趨向。

(二) 階段一之飼養與前置步驟

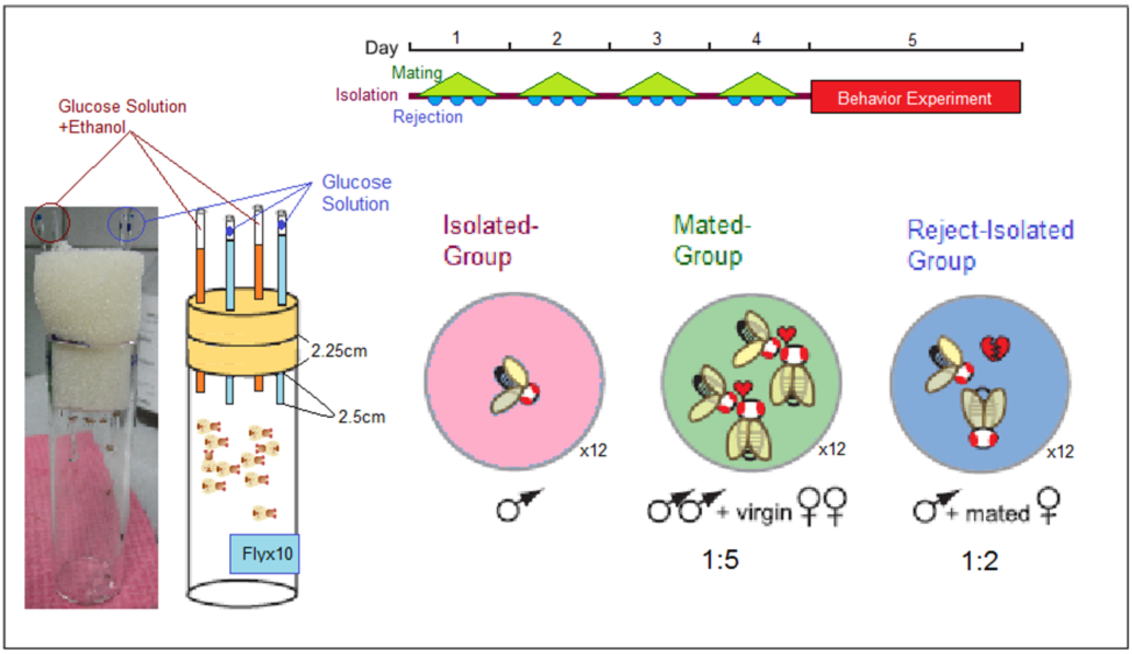


Fig.9 被拒絕公果蠅、成功交配公果蠅與對照組飼養流程

為了確立往後實驗是否具有可靠性，我們照著步驟重複文獻中的實驗。在實驗中，我們準備三組果蠅分別為 Rejected，Isolated，Mated，其中 Rejected 組天天以 1:2(公果蠅:交配過的母果蠅)的比例混合，讓公果蠅與交配過的母果蠅互動，造成求偶被拒的情況，每次一小時，一天三次，共為期四天。Mated 組一天一次以 1:5(公果蠅:處女果蠅)的比例混合，讓公果蠅與處女果蠅進行交配六小時，也是為期四天。Isolated 組則正常飼養四天。在這段時間，所有公果蠅皆分開飼養。後續實驗的飼養流程皆遵照這個流程。

階段一：文獻實驗的重複以及前置實驗

1. 用 15%的酒精來進行實驗，恰當嗎？

由文獻探討我們發現，公果蠅在自然界中最傾向食用 5%-15%酒精濃度之食物。為了增加吃了酒精之後，每組果蠅會產生的行為變化程度，我們希望使用 15%酒精濃度之食物。但為了確認文獻所述合理，我們自行設計一個實驗驗證其可信度。

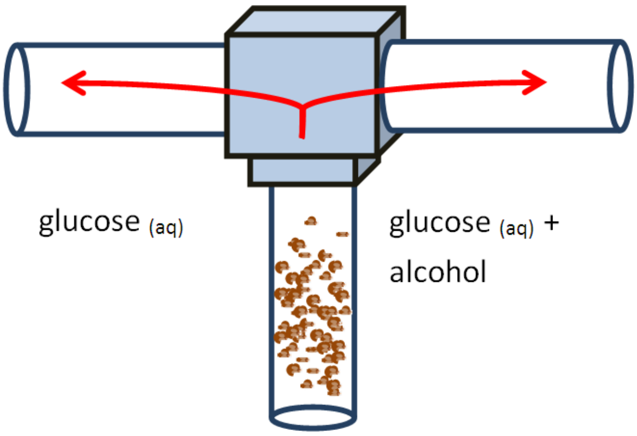


Fig.10 三向道使用示意圖

我們使用自行設計的 T 型迷宮，一邊擺置葡萄糖水，另一邊則每次擺放不同濃度的酒精葡萄糖糖水(自 100%遞減到 10%)，將 100 隻果蠅放置在下方的空管利用其背趨地性向上爬逼迫其選擇左右管內的食物，三

分鐘後紀錄其在酒精食物管中與正常食物管中之數量。為了確保實驗結果不會受趨光性或左右差異的影響，我們接著將左右邊調換重複第二次實驗。

2. **乙醚食物的濃度大約多少才恰當?**

因為我們也有使用到乙醚來進行實驗，所以我們重複了上一實驗步驟並把其中的酒精葡萄糖水改成乙醚葡萄糖水。

3. **公果蠅在求愛被拒絕後是否真的會提升酒精的攝食量？**

為了確認文獻的可信性與可重複性,我們跟著 science 期刊上關於「公果蠅求偶被拒」的實驗步驟，重複了數次實驗。

我們先從 Isolated、Mated、Rejected 三組各挑出十隻果蠅分別放入三個空管中，禁食二小時，以確保果蠅會食用所供給的飼料。接著在上面插入葡萄糖溶液以及含有 15%酒精的葡萄糖溶液各兩管，放置十小時，後以重量改變觀察各組所食用的量。由於實際減少的量難以用尺測量，我們先測量減少的重量(毫克)，再將其換算為體積(毫升)。

4. **公果蠅要花多久的時間才可從酒醉狀態回復?**

因為在實驗五中，我們測試了公果蠅攝食了含有酒精的葡萄糖水後的行為變化，所以我們必須觀測果蠅在攝食酒精之後，所產生的「酒醉」狀態大約會持續多久，以作為實驗五的參考標準。

第一組實驗中，我們養了八組每組五十隻的果蠅。分別餵食牠們酒精葡萄糖水 0(對照組)、1、2、4、6、8、12 以及 24 小時的時間。之後我們將所有果蠅放入長度為 15cm 的長管中，利用果蠅的背趨地性，紀錄該組果蠅進入了長管後，爬到頂端並停留兩分鐘的時間。

第二組實驗我們將以上八組(不同的八組)果蠅放入 15cm 的長管中，並記錄三十秒爬到管頂的隻數。測試時間為:在攝食完酒精食物後，每間隔一小時測一次，總計六次。

階段二: 酒精如何在被拒絕的公果蠅腦中產生作用？

5. **公果蠅在求偶被拒後，是否是「直接」想去食用酒精？**

果蠅食用含酒精的發酵水果是常見的現象，因此我們要確認果蠅求偶被拒後增加酒精的攝食，究竟是:

(1) 直接提高對酒精的趨向

(2) 求偶被拒後，經過吃了酒精後，腦中產生了某種作用回饋才增加酒精的攝食。

我們將飼養的 rejected 公果蠅大約兩百隻從食物管中取出放入空管，讓其不能攝食食物兩小時，然後把兩百隻公果蠅分成兩組。

第一組的公果蠅我們將其放入食物管中一小時後，直接把其放入 T 型迷宮下端，左右分別為葡萄糖水以及 15%酒精葡萄糖水，讓其選擇。三分鐘後記錄左右兩管分別有幾隻公果蠅。

第二組公果蠅我們讓它們食用正常食物與摻了酒精的食物各三十分鐘(與第一組的差別在於，第二組的公果蠅除了攝食了正常食物外，還攝食了摻了酒精的食物。) 將其放入 T 型迷宮下端，三分鐘後分別記錄左右管的隻數。兩組實驗各重複數次。

6. **公果蠅被拒絕後大腦下令的指令究竟是什麼?**

根據上一個實驗結果，求偶被拒的公果蠅居然會“排斥”含有酒精的食物,跟期刊上之求偶被拒的公果蠅攝食十二小時食物的結果完全相反。

於是我們推測:在被拒絕的同時，大腦應該不會直接下達「吃酒精」這個指令。於是我們比較了一般果蠅和求偶被拒之公果蠅對酒精的趨向。

我們飼養 Rejected 組與正常果蠅各 160 隻分成八組。接著將他們分別放入了 T 型迷宮讓牠們選擇酒精葡萄糖或葡萄糖水，三分鐘後將通道堵住，紀錄兩邊各有幾隻果蠅。最後比較兩組果蠅在選擇酒精葡萄糖水與葡萄糖水間比例之差異。

7. 公果蠅的求偶慾望會不會受到「求偶被拒」影響？

我們猜測，被拒絕後的公果蠅無論產生任何和改變，皆是為了增加其交配成功的機會。於是我們便比較了「求偶被拒」與「求偶被拒且攝食酒精」的公果蠅以及正常公果蠅與成功交配的公果蠅的求偶慾望。

我們飼養 Rejected、Mated、Isolated 以及 Rejected-ethanol 組各十隻進行實驗。實驗的前置飼養步驟如下：

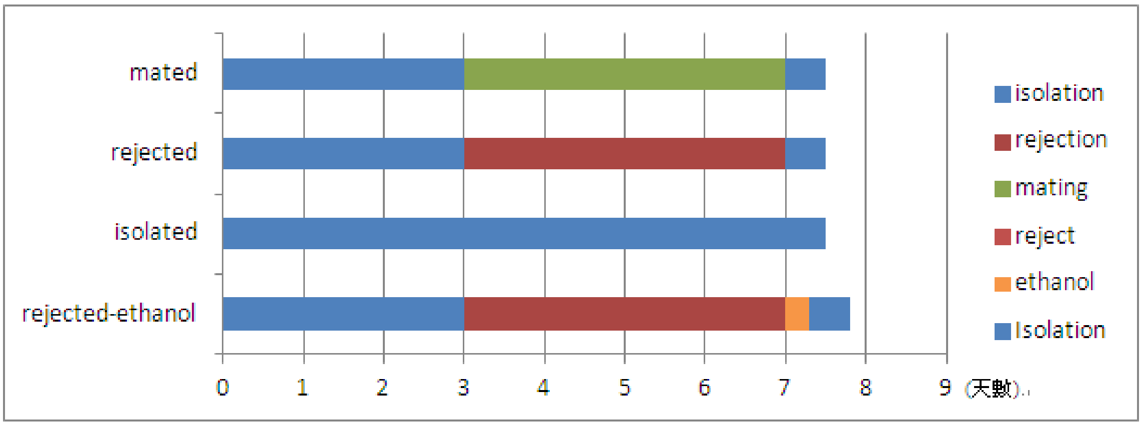


Fig11　此實驗之果蠅飼養流程 mated: mated 組

(1) Mated: mated 組
(2) Rejected: rejected 組
(3) Isolated: isolated 組
(4) Reject-ethanol:同 Rejected 組，但在將與母果蠅放置前會先餵食其酒精食物一小時。

將四組公果蠅在七天飼養期後，靜置四小時，再分別與一隻處女果蠅置入求偶房中，紀錄公果蠅開始追求母果蠅的時間(秒數)。

8. **被拒絕的公果蠅，也會增加對乙醚食物的攝食量嗎?**

我們不知道公果蠅在被拒絕後，會去食用含酒精之食物，是出自於「酒精能阻卻神經傳導使求偶被拒後腦中產生的感覺被鎮定」，或者為「酒精使公果蠅腦中產生了"增加酒精攝食"的訊號」。為了得知是哪個推論造成了被拒絕之公果蠅提升對酒精的攝取量，我們嘗試使用同樣能阻礙神經傳導的乙醚，看被拒絕之公果蠅對乙醚之攝食量是否也是較高的。

此實驗之步驟與前一實驗(三)「被拒絕之公果蠅酒精食物攝取量實驗」相同，但是將酒精替換成乙醚，並且根據前置實驗(二)，將乙醚濃度設定在 10%，後觀察果蠅的食用量。

(三) 階段三之前置飼養步驟

階段三的實驗是想要了解「NPF 在腦中的量」與「公果蠅求偶被拒之後產生之行為」的關聯性，因此除了原本的三組果蠅，我們另外飼養了五組利用 Gal4-UAS 系統配製而成的果蠅。而與原本飼養唯一不同的地方是，這次飼養全程維持在 30 度的溫度。(為了使基因工具 UAS-kir2.1^{ts} 以及 UAS-dTrpa1 產生作用)

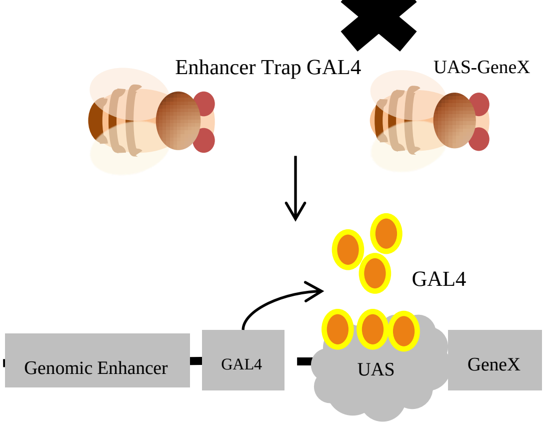


Fig12:左邊的公果蠅攜帶著 Gal4 基因，右邊的母果蠅攜帶著 UAS 以及其後接著的 gene X。於是其子代便會同時擁有 Gal4+UAS-gene X。Gal4 基因所製造出的蛋白質如鑰匙，可以開啟 UAS 以及讓其背後的 gene X 的蛋白質大量製造，影響標定在 GAL4 的序號。

1. NPF-Gal4+UAS-kir2.1

UAS-kir2.1:此基因工具會增加標定神經細胞的鉀離子通道反應率，使同時攜帶 Gal4+UAS-gene X 的神經細胞無法被去極化。進而使該 gene X 不表現。(Baines et al., 2001;Paradis et al., 2001)

這株果蠅在 UAS 上所配置的基因 kir2.1 在溫度到達 28 度以上時,會抑制NPF的神經細胞表現。因此我們可以讓公果蠅在不被拒絕的情況下，使腦中 NPF 神經的活性降低。

2. NPF-Gal4+UAS-dTrpA1

UAS-dTrpA1: 此基因工具的功能也是調控鉀離子通道的反應率，但是相反的，它會抑制鉀離子通道的反應，使同時攜帶 Gal4+UAS 的細胞容易被去極化。 (Rosenzweig et al., 2005;Rosenzweig et al., 2008)

與上一株果蠅相同，dTrpA1 所製造出的是一種可以利用溫度調控的蛋白。不過它的功用和 kir2.1 相反，在溫度到達 28 度以上時，會使分泌 NPF 的神經細胞的活性被抑制。

3. NPF-Gal4+ w1118

4. UAS-dTrpA1+w1118

5. UAS-kir2.1+w1118

以上三組(三-五組)果蠅是為了確認果蠅行為上的改變並不是因為擁有單一一個基因(若基因中只有 Gal4 或只有 UAS，其並不會作用，也沒辦法控制 NPF 的表現量)而造成，若行為的控制皆是因為會 NPF 的神經的活性的改變，則這三組應該會和對照組 w1118(isolated group)有類似的行為模式。

階段三：NPF 神經的活性變化是否為被拒絕果蠅行為改變之主因？

此階段的實驗利用上一飼養步驟飼養出來的八組公果蠅(原本三組以及 Gal4-UAS system 的五組)測試了以下三個實驗:

9. **腦中 NPF 神經活性改變是否影響公果蠅對酒精之攝取量?**

10. **腦中 NPF 神經活性改變是否影響公果蠅對酒精之趨向?**

11. **腦中 NPF 神經活性改變是否影響公果蠅的求偶慾望?**

後續再進行數據分析，可了解 NPF 神經活性的改變究竟是否為公果蠅被拒絕之後行為改變的主因。

但是因為即便 NPF 神經的活性被調整仍舊不能確定腦中 NPF 的量被改變，於是我們做了以下兩個實驗來確定，NPF 神經的活性的確會影響 NPF 在腦中的存量。

12. **NPF 在腦中那裡作用?**

    此實驗利用 Gal4-UAS 系統配了兩株果蠅，亦即:
    NPF-Gal4+5137；5130 以及 NPFr-Gal4+5137；5130

    5137；5130 是一種 UAS 後端會接螢光蛋白的果蠅，因此在與 NPF-Gal4 作用後會在 NPF 被產生的地方做出螢光蛋白。我們便可利用螢光顯微鏡觀察 NPF 在果蠅大腦的分布情形。而 NPFr 指的是 NPF receptor，即為神經傳導物 NPF 的接收器。在利用細鑷子剖出果蠅大腦後，使用共軛焦螢光顯微鏡比對 NPF 以及 NPFr 的分佈，便可找出 NPF 在大腦中作用的位置。

13. **NPF 神經的活性變化是否會影響腦中 NPF 的量**

為了看出 NPF 神經活性的變化到底會不會造成腦內 NPF 量的變化，我們使用 NPF 的螢光抗體(antibody) 來標定以下五組公果蠅腦中 NPF 的量。

首先我們分別飼養五組公果蠅

(1) Rejected

(2) Isolated

(3) Mated

(4) Gal4-NPF+UAS-kir2,1

(5) Gal4-NPF+UAS-dTrpa1

在飼養流程結束後，我們將他們冰凍麻醉，並剖出他們的腦浸泡在 NPF 的螢光抗體中。之後就拿到共軛焦螢光顯微鏡下掃圖，即可得到果蠅腦中 NPF 量的影像。

階段四: 探討求偶被拒絕的公果蠅的行為改變是否符合「類-情緒行為」的定義

被拒絕的公果蠅在被母果蠅拒絕並吃過酒精後，會提高對酒精的趨性，引起了我們懷疑，情緒在這神經系統較人類簡單許多的模式生物的行為中，是否扮演著驅動的功能?

閱讀了《Two Different Forms of Arousal in Drosophila Are Oppositely Regulated by the Dopamine D1 Receptor Ortholog DopR via Distinct Neural Circuits》這篇文章後，其中談到了「類-情緒驅動行為」“emotion-like behavior”須具備的三樣條件:

Persistent(刺激後反應持續)

Graduation of intensity(不同程度刺激造成不同程度行為改變)

Positive and negative reactions(正面 or 負面的選擇，討論此情緒為正面或負面情緒。)

就是這三個條件區分了情緒行為與簡單反射行為(simple-reflex behavior)的差異。於是我們便設計了以下實驗來觀察，公果蠅在被拒絕後的行為反應是否是符合這三條件。

14. **公果蠅在被拒絕後，其行為改變是否有持續性?(persistent)**

我們將被拒絕的公果蠅在為期四天的 rejected 後，靜置一天至三天，觀察其行為之改變是否延續。

15. **不同的拒絕程度，是否讓公果蠅產生不同程度的行為改變?(graduation of intensity)**

此實驗中，我們將公果蠅分成三組，分別拒絕一天、兩天、四天，觀察其行為是否隨著其被拒絕的天數增長，而有更明顯的改變。

參、　研究結果與討論

階段一：文獻實驗的重複以及前置實驗

1. **將酒精濃度設定為 15%是恰當的。**

為了確認大約多少濃度的酒精葡萄糖水對我們來說是最恰當進行實驗的，我們將不同組的公果蠅放入左邊為不同濃度之酒精葡萄糖水、右邊為一般葡萄糖水的三向道，並觀察各組公果蠅選擇兩食物之比例。

從表中可得知，公果蠅會迴避高濃度的酒精，當濃度介於 10~15%之間時，公果蠅對各種食物的偏好最接近一比一。根據此實驗的結果，選擇十五%酒精濃度之葡萄糖水是合理的，根據兩個挑選標準:

(1) 正常公果蠅對此濃度之酒精葡萄糖水不會迴避

(2) 盡量選擇一個公果蠅在自然界中熟悉的酒精食物濃度的最高值(為了讓各組公果蠅在攝食食物後，差異明顯。)

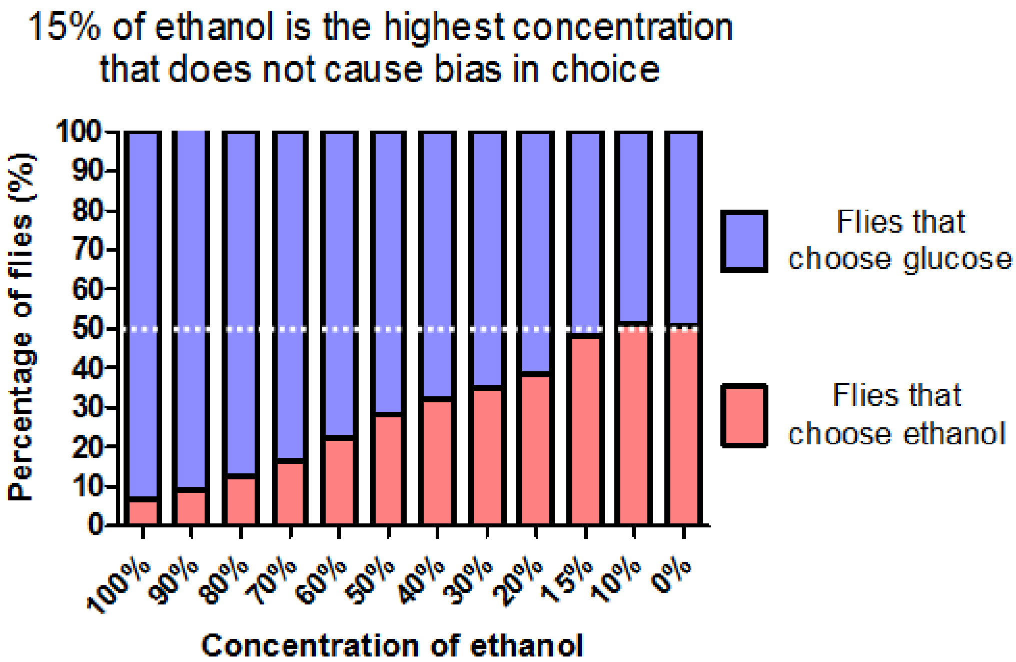


Fig.13 果蠅對不同濃度酒精葡萄糖的選擇

2. **根據我們的實驗結果，將乙醚濃度設定在 10%**

與上一實驗相同的理由，我們需要知道多少濃度的乙醚葡萄糖水是恰當進行實驗的。於是我們重覆上一實驗之步驟，但是將其中的酒精換成乙醚，後觀察各組選擇兩食物之比例。

由表中可得知，公果蠅會迴避較高濃度的乙醚葡萄糖，而當濃度介於 0~15%時，公果蠅選擇兩種食物的比例最接近一比一。但是因為我們觀察當乙醚濃度為 15%時，所有在三向道內跑向乙醚食物的公果蠅皆會因為乙醚蒸氣而失去行動能力，所以我們最終選擇的乙醚濃度為 10%。

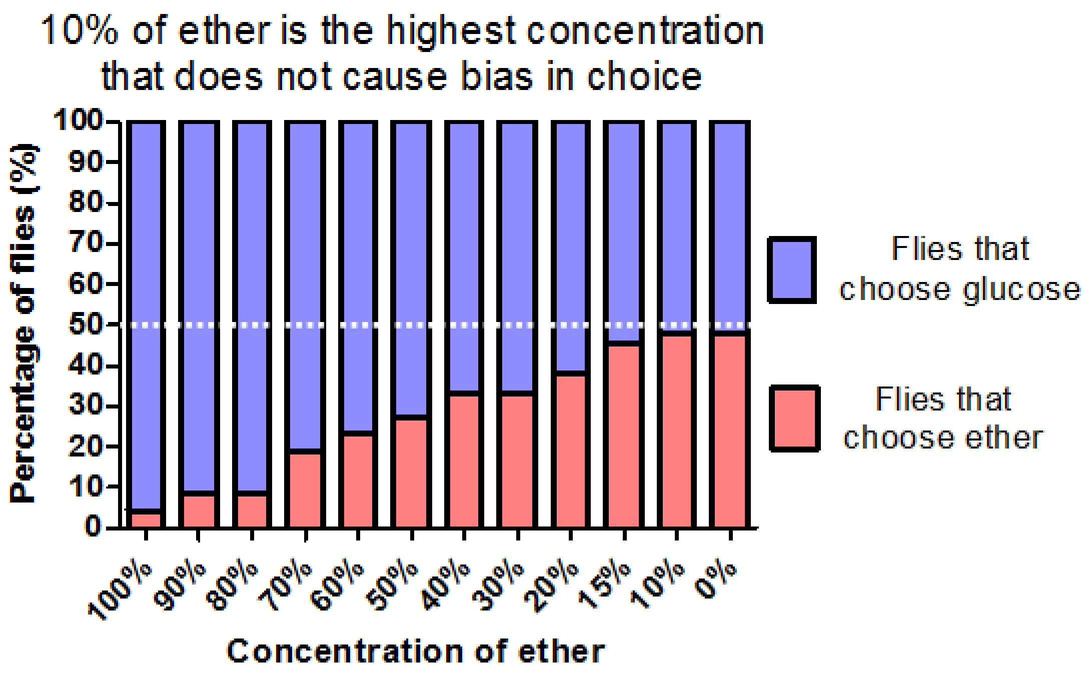


Fig.14 果蠅對不同濃度酒精葡萄糖的選擇 (%)

3. **求偶被拒的公果蠅會明顯提升含酒精食物攝取比例**

為了確認文獻的可信性與可重複性，我們跟著 science 期刊上關於「公果蠅求偶被拒」的實驗步驟，重複了數次實驗。首先我們先將三種公果蠅(rejected, isolated, mated)飼養出來後，將他們放入插著酒精葡萄糖以及

葡萄糖水的毛細管，觀察他們在二十四小時內，分別攝食了多少酒精食物以及正常食物。

從圖表中可看出，三組公果蠅的攝食總量並沒有太大的差異。而被拒絕的公果蠅(rejected)比正常交配(mated)以及單獨飼養(isolated)的公果蠅更為偏好酒精食物。此結果不但印證文獻上指出的「被拒絕的公果蠅會提升酒精的攝食量」，也發現若只有純粹的隔離飼養(isolated group)不使其遭到拒絕，其攝食酒精的 PI 值與「成功交配」組的並沒有太大差異。

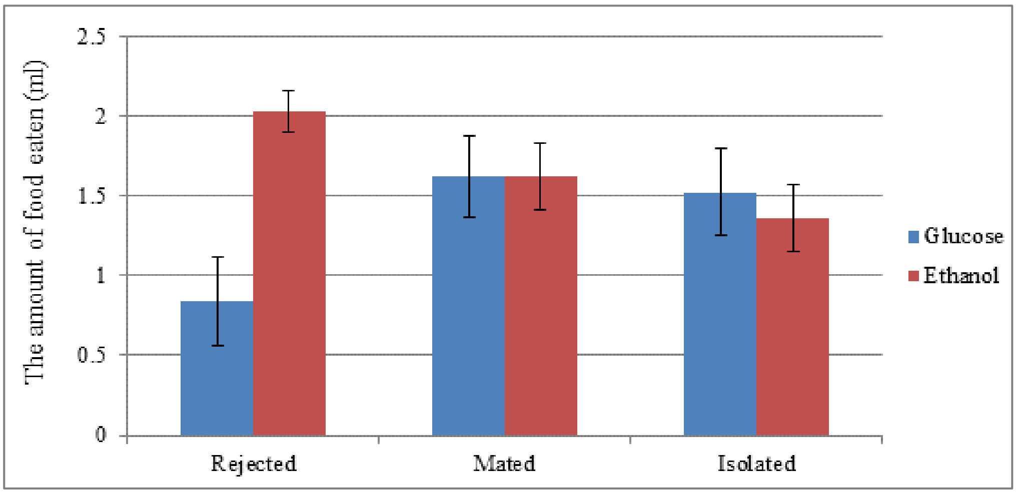


Fig.15 酒精實驗三組果蠅之食用量

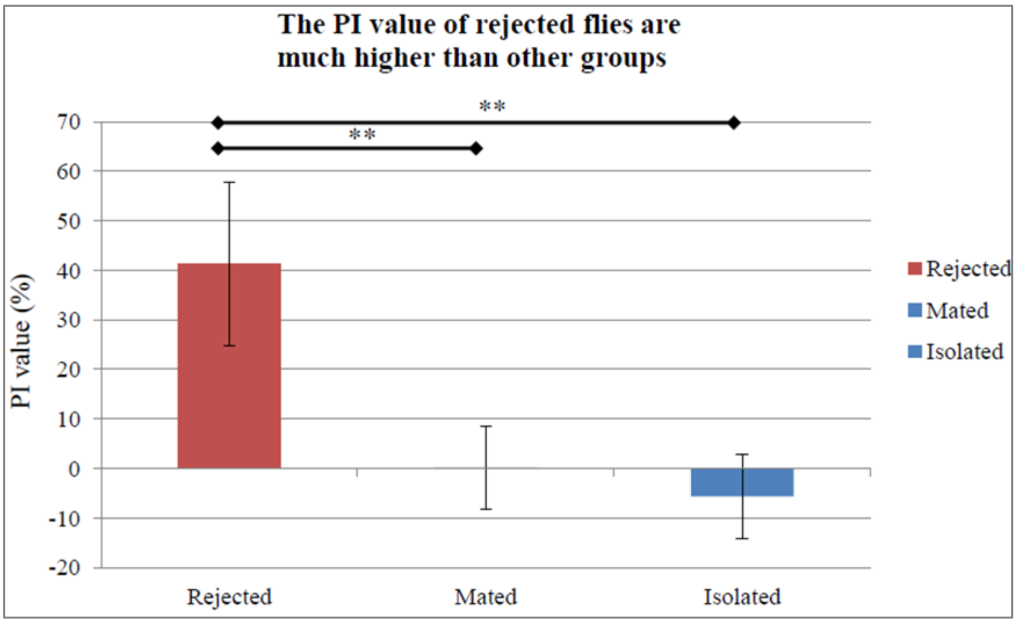


Fig.16 三組果蠅之 PI 值((酒精食物量-正常食物量)/(酒精食物量+正常食物量))

4. **攝食一小時酒精食物之公果蠅大約要花四小時來回復爬行速度**

因為在後面的實驗中，我們會需要測試公果蠅在攝食酒精之後的行為，所以我們要確定其在攝食酒精食物之後，需要花多少時間來回復行為能力。於是我們便將攝食不同時間的酒精食物的果蠅放入 15cm 的管子中，觀察牠們要花幾個小時才能與沒有攝食酒精食物的公果蠅的爬上 15cm 管子的比例是一樣的。

實驗結果指出:攝食一個小時的公果蠅在 1~4 小時中在 30s 內爬上 15cm 管子的隻數皆低於 90%，超過四個小時後，其爬行到管頂的隻數才會跟沒有吃過酒精的組別一樣。根據這個結果，之後我們的 rejected-ethanol 組皆是在將其放置四小時之後，才進行測試。

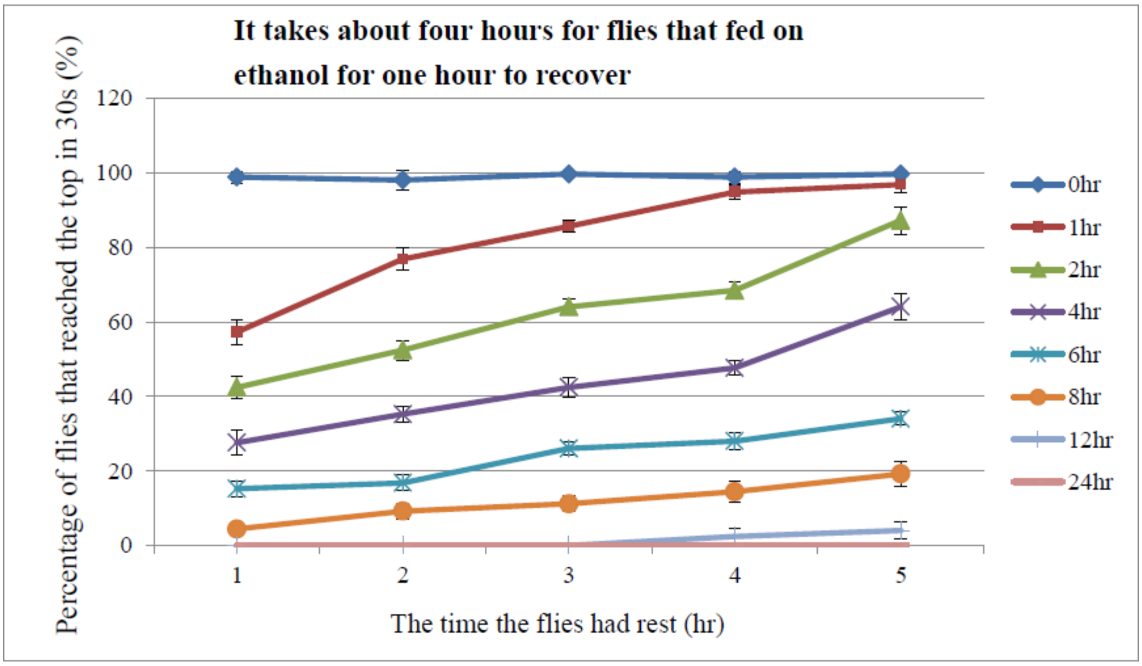


Fig.17 攝食酒精的公果蠅，要花多少時間才能回復?

階段一前置實驗的統整結果:

(1) 酒精葡萄糖水的濃度:15%
(2) 乙醚葡萄糖水的濃度:10%
(3) 求偶被拒絕的公果蠅的確會增加酒精的攝食量!
(4) 之後如果公果蠅要在吃完酒精後進行行為測試，其流程皆為:攝食酒精一小時後，放置四個小時，再進行行為測試。

階段二:酒精如何在被拒絕的公果蠅腦中產生作用？

5. **公果蠅被拒絕後並不會馬上提高酒精趨向，而是在吃了酒精之後才提高對酒精之趨向(下圖 Fig.18，AB 組互相比較)**

因為果蠅食用含酒精的發酵水果是常見的現象，因此我們要確認果蠅求偶被拒後，在 12hr 內增加酒精的攝食，究竟是:1. 求偶被拒後，直接提高對酒精的趨向 2. 求偶被拒後，經過吃了酒精後，腦中產生了某種作用回饋 ，才增加酒精的攝食。

於是我們便將求偶被拒之公果蠅分成兩組，第一組將其直接放入三向道中選擇，而第二組則是餵食了含有酒精食物後，才放入三向道中讓其選擇。後記錄其對酒精之趨向(趨向指的是三分鐘內的食物選擇，而攝食量則是 12hr 內的總攝食量。)

(比較下圖的 A 以及 B)(A 為求偶被拒後**無**攝食酒精便直接放入三向道之公果蠅；B 為求偶被拒後，攝食酒精後才放入三向道之公果蠅)

實驗結果顯示:被拒絕的果蠅在尚未攝食酒精前進入 T 型管時，大多數皆是往葡萄糖水移動(A)；然而被拒絕的公果蠅在食用過摻了酒精的葡萄糖水後，選擇摻了酒精葡萄糖水的比例增加了(B)。這兩個測試的差異使我們判定公果蠅在被拒絕之後提高對酒精的攝食量的原因是出自於:被拒絕之後，酒精在其腦內產生的回饋。而非求偶被拒後直接增加對酒精之趨性。

此實驗與前一個實驗(實驗三)最主要的差異在於:此實驗是在測試求偶被拒的公果蠅“短期”對酒精的趨向，上一個實驗則是探討求偶被拒的公果蠅 12HR 對食物的總攝食量。

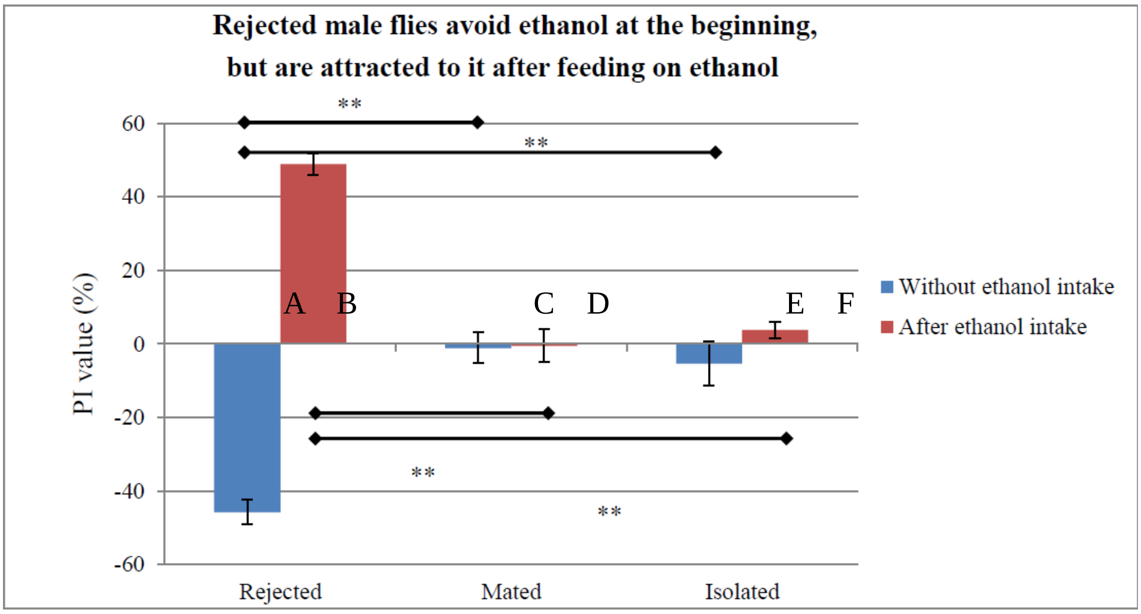


Fig.18　無吃過酒精之公果蠅與吃過酒精之公果蠅對酒精趨向差異

6. **相較於成功交配以及對照組之公果蠅，求偶被拒的公果蠅會明顯迴避含有酒精食物(Fig 18:A 組與 C,E 組比較)**

根據上一個實驗的結果，求偶被拒的公果蠅居然會“排斥”含有酒精的食物，跟期刊上之求偶被拒公果蠅攝食十二小時食物的結果完全相反。於是我們推測:在被拒絕的同時，大腦應該不會直接下達「吃酒精」這個指令。於是我們比較了一般果蠅(E)以及成功交配之公果蠅(C)和求偶被拒之公果蠅(A)在三分鐘內，對酒精的趨向。

實驗的結果顯示，成功交配之公果蠅以及一般公果蠅對正常食物與含酒精食物趨向差異不大(C,E 組)，但是求偶被拒之公果蠅在尚未攝食酒精前明顯會去迴避含酒精食物(A)。

7. **公果蠅被拒絕後會更積極追求母果蠅，吃過酒精卻變消極**

我們猜測，被拒絕後的公果蠅無論其行為產生任何改變，皆是為了增加其成功交配的機會，於是我們推測，求偶被拒的公果蠅可能會增加

其求偶慾望。此實驗中我們比較了「求偶被拒」與「求偶被拒且攝食酒精」的公果蠅以及正常公果蠅與成功交配的公果蠅的求偶慾望。

從數據中可看出，被拒絕的公果蠅確實比成功交配的公果蠅還要更積極的去追求其他母果蠅，除此之外，若將食用酒精後之求偶被拒公果蠅，放入求偶房中，其對母果蠅的求偶慾望反而比正常交配的果蠅還要更低。我們推測原因是出自於，酒精會降低其神經系統效能，造成公果蠅在接收到母果蠅氣味時無追求行為產生。

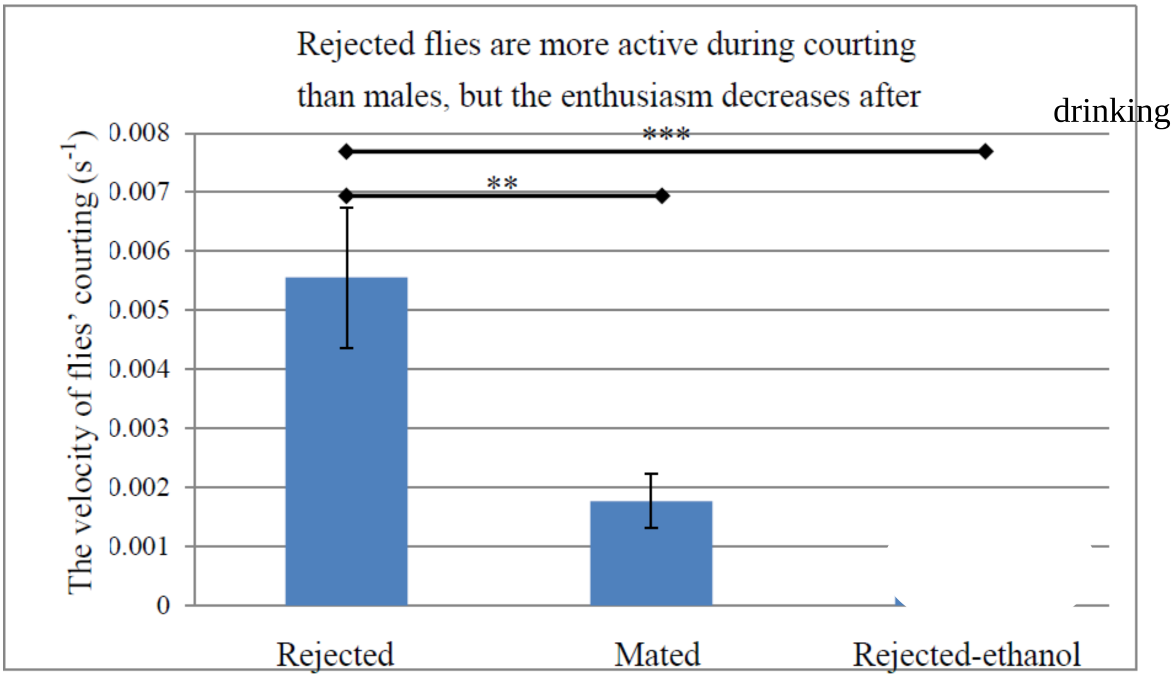


Fig.19 不同組別之果蠅對母果蠅求偶所需時間的差別

(reject-組:reject 組在與雌果蠅一同放置前未攝食酒精。
reject-ethanol 組:reject 組在與雌果蠅一同放置前，先餵食酒精食物)

8. **酒精的作用可能是阻斷信號**

我們不知道公果蠅在求偶被拒後，會去食用含酒精之食物，是出自於「酒精能阻卻神經傳導使求偶被拒後腦中產生的感覺被鎮定」，或者為「酒精使公果蠅腦中產生了“增加酒精攝食”的訊號」。為了得知是哪個推論造成了被拒絕之公果蠅提升對酒精的攝取量，我們嘗試使用同樣能

阻礙神經傳導的乙醚，看被拒絕之公果蠅對乙醚之攝食量是否也是較高的。此實驗中只把實驗三的步驟修改，並且把其中的酒精改成乙醚。

實驗結果顯示，求偶被拒的公果蠅攝食乙醚葡萄糖的比例較其他組高。我們推測求偶被拒的公果蠅提高酒精攝食量原因是出自於酒精與乙醚共有的“非選擇性神經鎮定”功能。

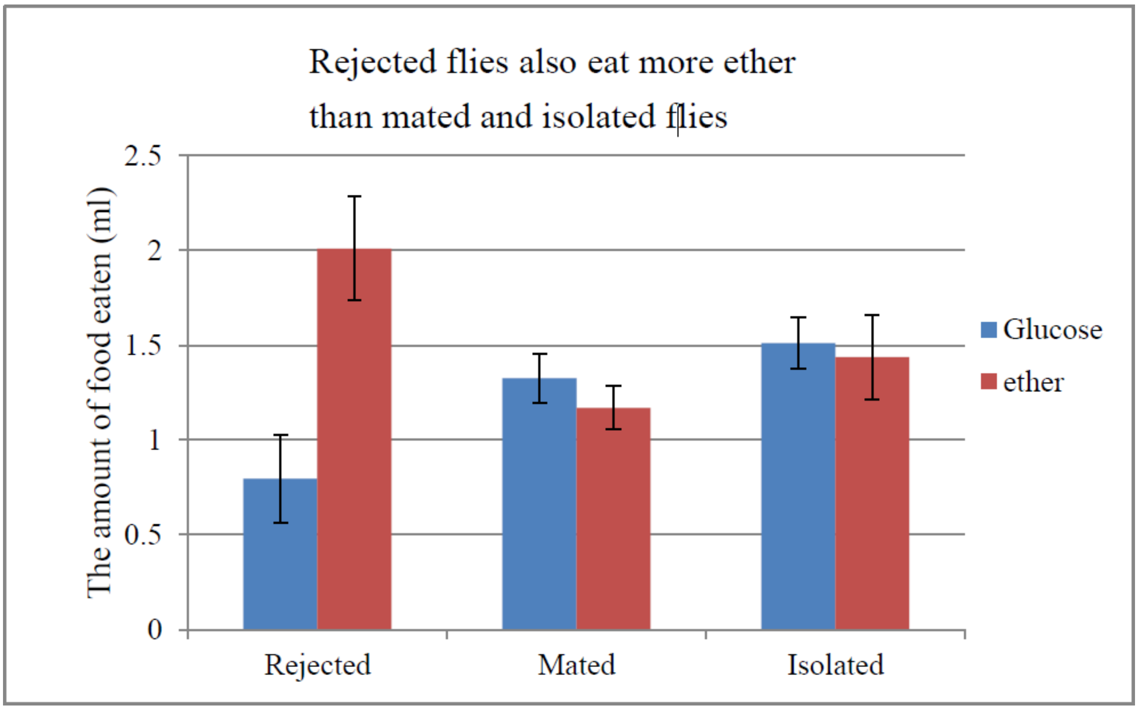


Fig.20 三組公果蠅對兩種食物的攝食量

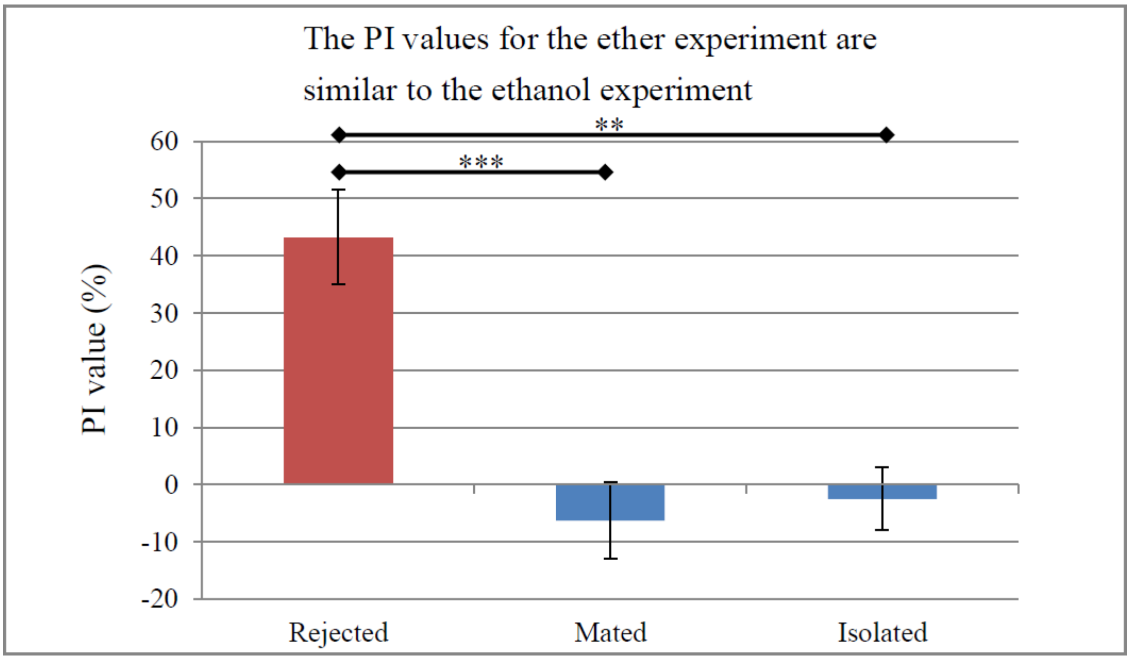


Fig21. 三組公果蠅對乙醚食物 PI

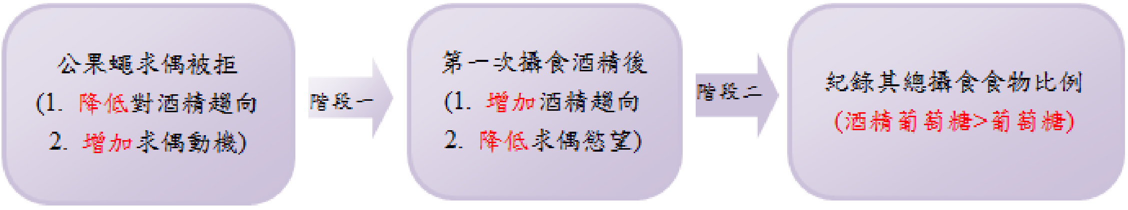


Fig.22 階段二之實驗結果統整

階段三：神經傳導物質 NPF 對公果蠅行為的影響為何？

9. NPF 降低是攝食行為改變之主因！(12hr 的攝取量)

此實驗的目的為觀察 NPF 神經活性增減是否會造成公果蠅酒精攝食量的變化。於是在培養出原本的三組公果蠅(rejected, isolated, mated)以及 UAS-Gal4 系統的五組(NPF-Gal4+UAS-kir2.1, NPF-Gal4+UAS-dTrpA1, NPF-Gal4+ w1118, UAS-dTrpA1+w1118, UAS-kir2.1+w1118)後，我們便將他們放入上面插有毛細管食物的空管中，觀察牠們 12hr 內對兩種食物的攝食量。

從 PI 值圖中可看出，Rejected 組及 NPF-Gal4+UAS-kir2.1(NPF 的神經活性被抑制)相較於其他組，攝食酒精的比例較高。

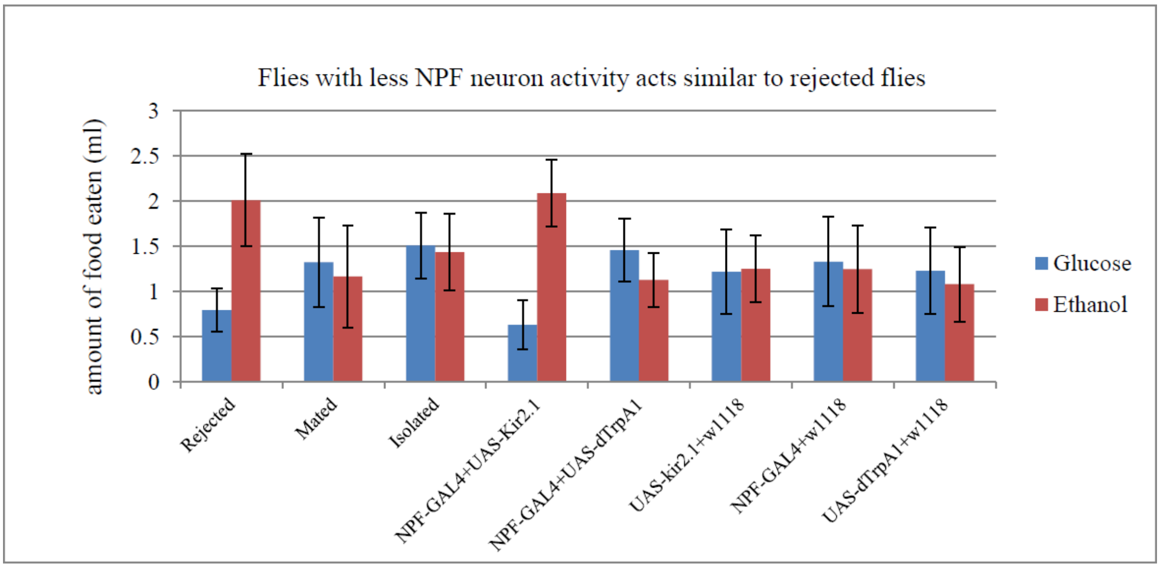


Fig.23 各組果蠅對酒精糖水以及糖水之攝食量

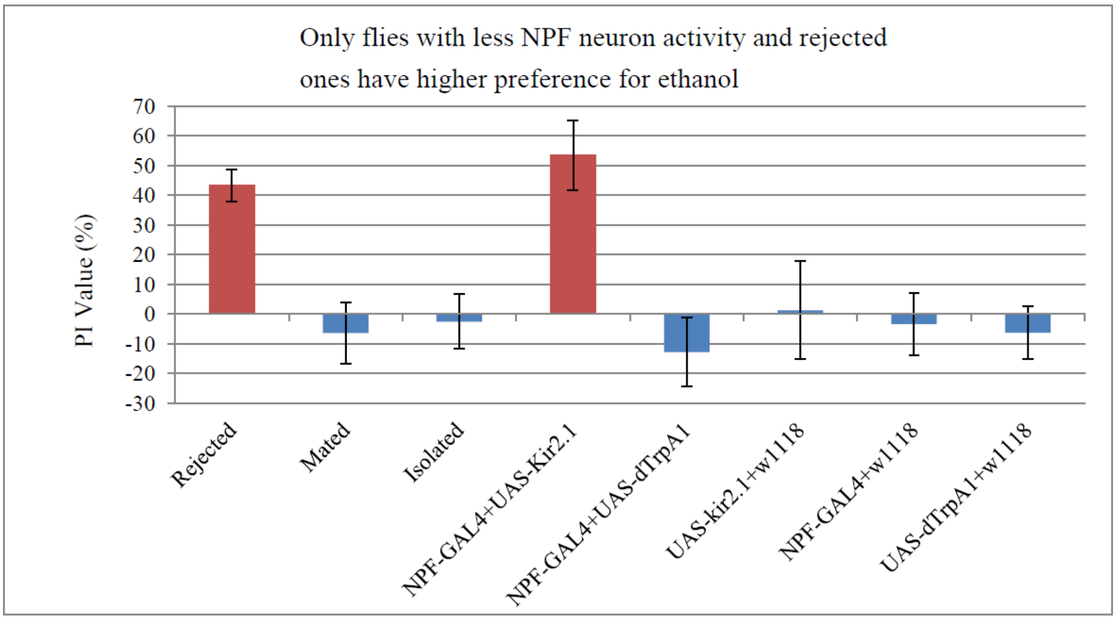


Fig.24 實驗八個組之 PI 值

10. **NPF 降低會使果蠅遠離酒精！(三分鐘的選擇時間)(加入 12hr)**

此實驗的目的為觀察 NPF 神經活性被調控的公果蠅，對酒精的趨向是否會改變。於是我們在飼養八組公果蠅的流程結束後，將八組公果蠅放入三向道中，觀察他們在三分鐘內選擇兩種食物的比例。

從圖中可看出，雖然不比 Reject 組，但 NPF-Gal4+UAS-kir2.1 在三分鐘的選擇中仍較其他組排斥酒精。而 NPF-Gal4+UAS-dTrpA1 組與正常公果蠅並沒有明顯差異。

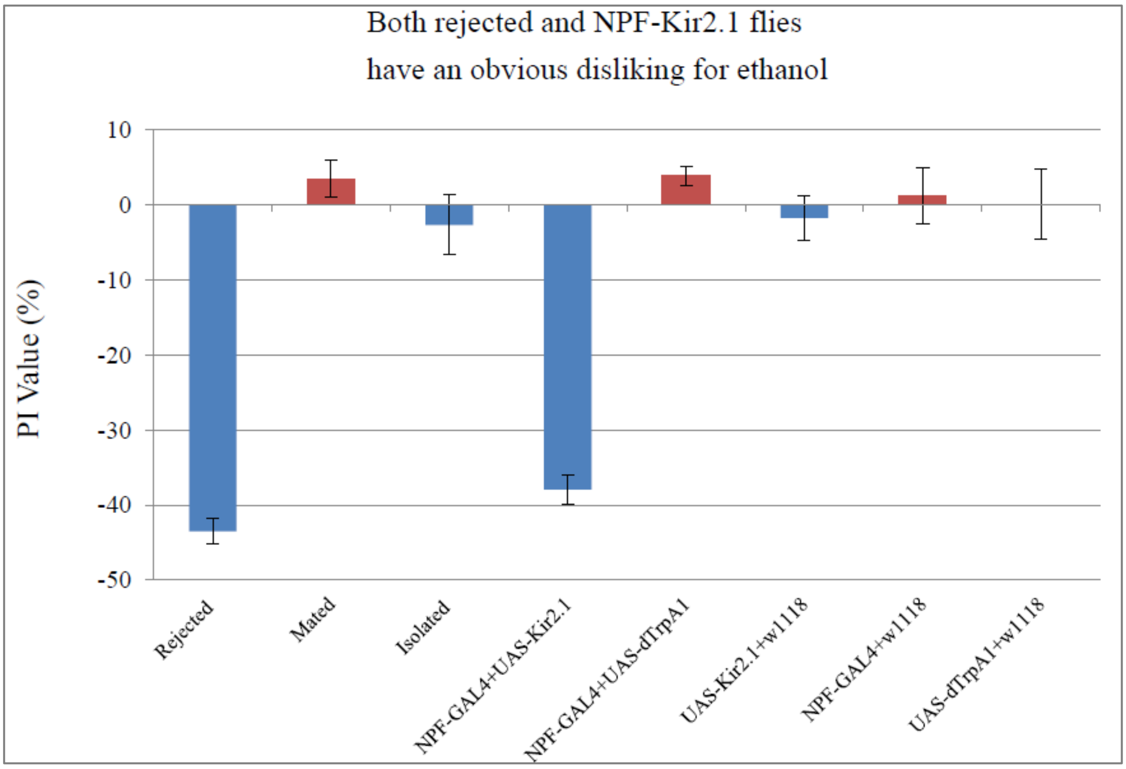


Fig. 25 各組果蠅對酒精之趨向比對

11. **NPF 量降低會影響公果蠅的求偶慾望**

公果蠅在被母果蠅拒絕之後，其對母果蠅的求偶慾望便會提升，引起我的好奇，NPF 會不會是其中的控制因子呢?於是在八組的飼養流程過後，我們把公果蠅放放入求偶房中觀察其求偶的慾望強度。

由圖可以發現，NPF-Gal4+UAS-kir2.1 的公果蠅(公果蠅腦中 NFF 神經活性被抑制)的求偶慾望是所有組中最強的，而 NPF-Gal4+UAS-dTrpA1 組的公果蠅(公果蠅腦中 NPF 神經活性增加)跟 mate 組的公果蠅的求偶動機較接近。由這個結果我們可以推知:被拒絕的公果蠅的求偶動機上升可能就是被 NPF 神經的或活性來控制。

但是，我們並不知道，NPF 神經的活性的變化是否可以造成 NPF 的量的變化，下一個實驗就是要探討 NPF 神經的活性變化可否造成 NPF 量的變化。

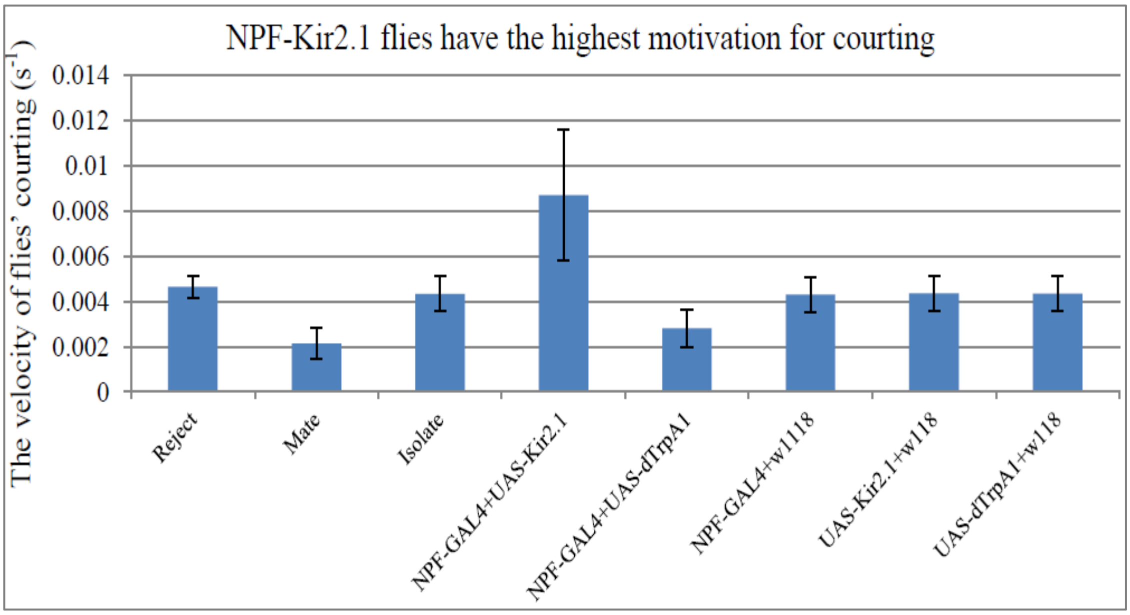


Fig.26 各組公果蠅的求偶動機

12. **NPF 分布區域**

此實驗利用 Gal4-UAS 系統配了兩株果蠅，亦即:NPF-Gal4+5137；5130 以及 NPFr-Gal4+5137；5130 來尋找 NPF 的在腦中的分泌位置以及 NPF 的接收位置。

由兩張圖的比較可以發現 NPF 分泌與其接受器主要重疊部分分布於中央以及後方腦區。

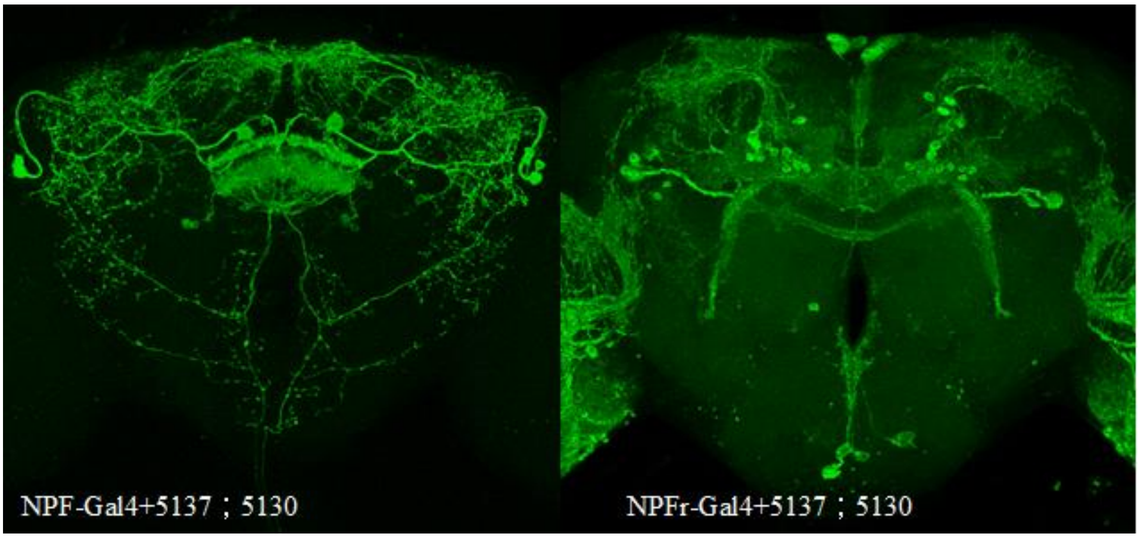


Fig.27 左圖為 NPF 分布，右圖為 NPFr 分布

13. **NPF 神經活性變化的確會影響腦中 NPF 的量!**

為了看出 NPF 神經活性的變化到底會不會造成腦內 NPF 量的變化，我們使用 NPF 的螢光抗體(antibody) 來標定以下公果蠅腦中 NPF 的量。

由實驗結果(Fig30 Fig31)可以看出，NPF 神經活性被激發的公果蠅，腦中產生的 NPF 的確比較多，而 NPF 神經活性被抑制的公果蠅，NPF 的量比較少。根據這個階段的實驗結果，我們可以證明:果蠅腦中 NPF 的量的變化的確會影響公果蠅對酒精的攝食量、對酒精趨向以及求偶慾望。

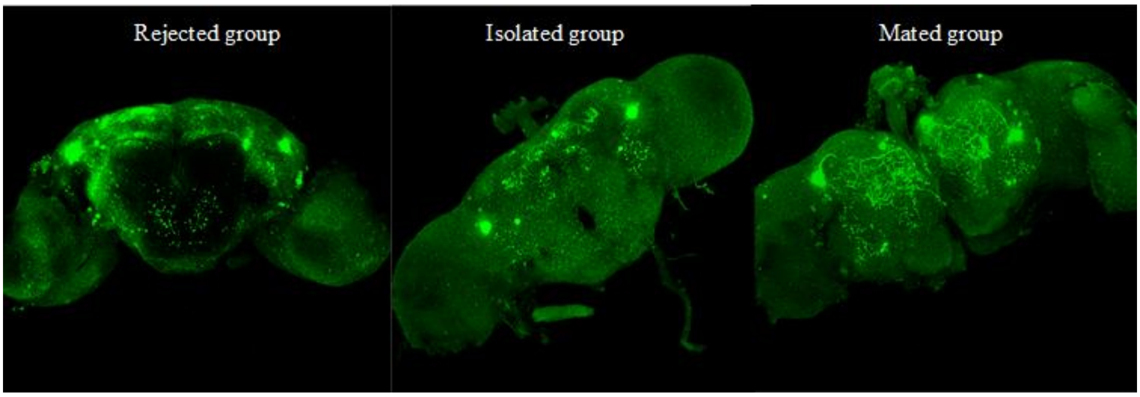


Fig.28 NPF 神經活性變化與 NPF 的量比較圖
(左 rejected、中為 isolated、右為 mated 之 NPF 螢光蛋白圖)

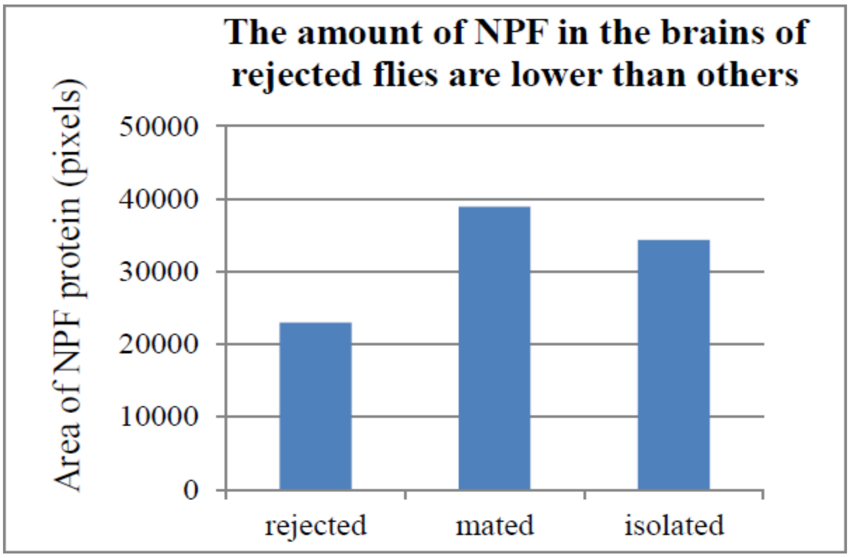


Fig.29 三組 NPF 螢光蛋白發光之像素比較表

由像素分析圖中可以發現，被拒絕的公果蠅腦中分泌 NPF 的量的確比其他兩組還要少。

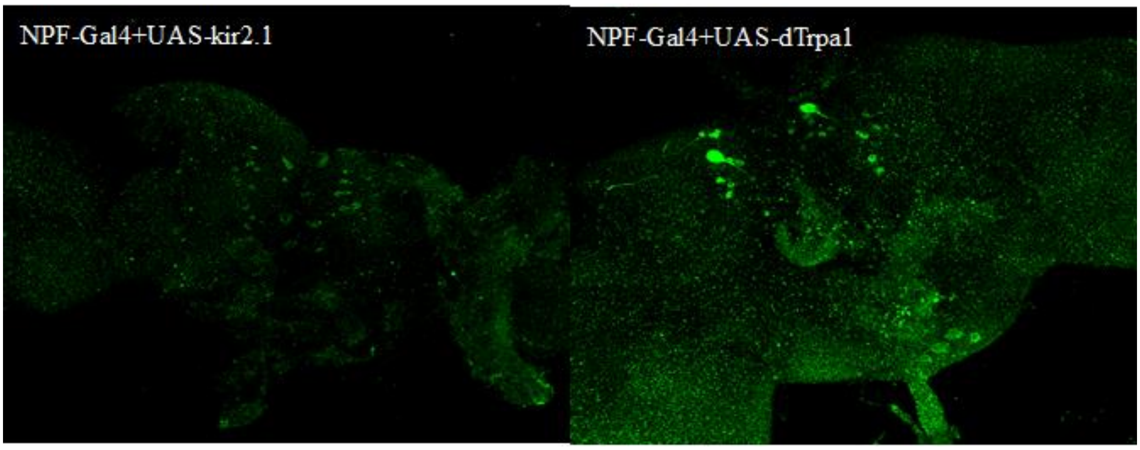


Fig.30 NPF 神經活性變化與 NPF 的量比較圖

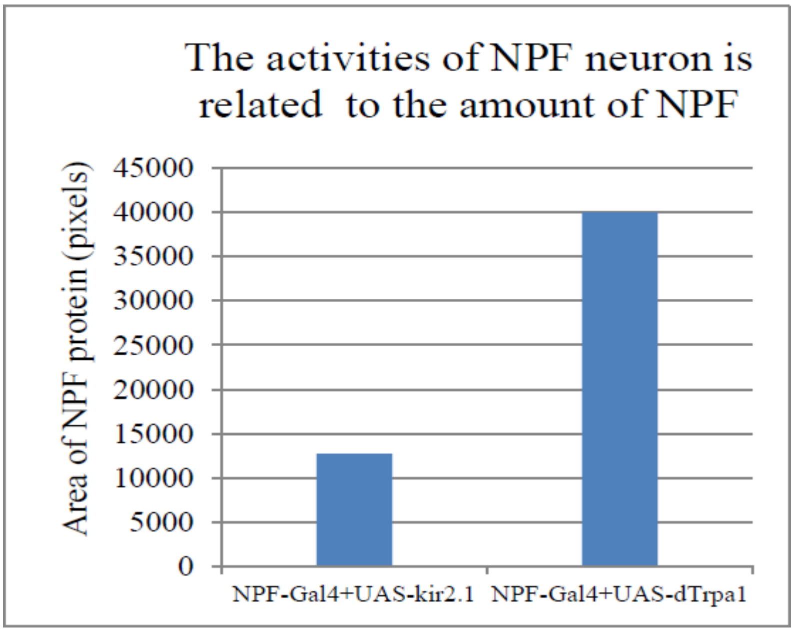


Fig.31 NPF 神經活性之 NPF 胜肽發光之像素比較表

階段四：探討求偶被拒絕的公果蠅的行為改變是否符合「類-情緒行為」的定義

14. 被拒絕的公果蠅，其被改變行為的確有持續性!

我們將被拒絕的公果蠅在為期四天的 rejected 後，靜置一天至三天，再將其放入三向管中 3 分鐘，觀察其對酒精趨性之改變是否延續。

由圖表可知，公果蠅在求偶被拒後，其「對酒精的趨向會比正常公果蠅還要低」這樣的行為改變，可以持續三天，由此可以推知，被拒絕後的行為改變是有持續性的，有別於一般「簡單、反射，刺激反應行為(simple, reflexive stimuli-response)

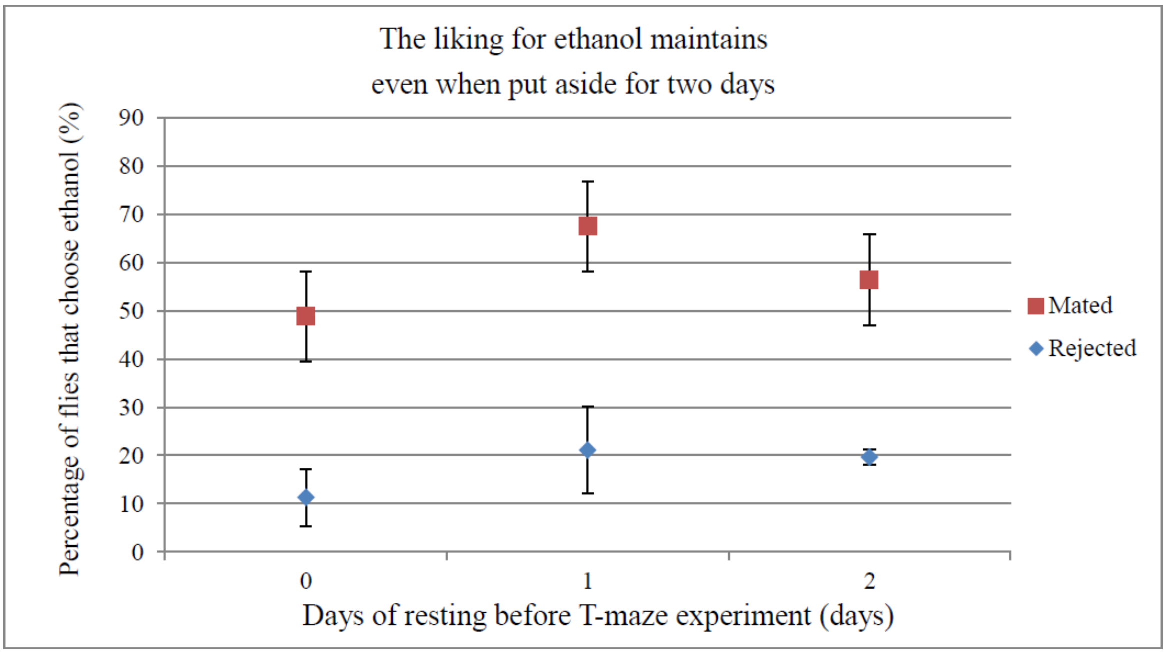


Fig.32，公果蠅被拒絕後行為改變是否有持續性?

15. 不同的拒絕程度，會產生不同的行為改變!(此實驗中，將公果蠅分別被拒絕一天、兩天以及四天後，測試其對酒精之趨向)

此實驗中，我們將公果蠅分成三組，分別拒絕一天、兩天、四天，再將其放入三向管中三分中，觀察其在不同拒絕天數，趨向的改變是否隨著其被拒絕的天數增長，而有更明顯的改變。

由圖表可以發現，隨著拒絕的天數增加，公果蠅在選擇摻了酒精的食物的比率會跟著下降。這個結果符合類情緒行為所必須符合的條件二:隨著刺激強度增加，反應改變程度越大。(Graduation of intensity)

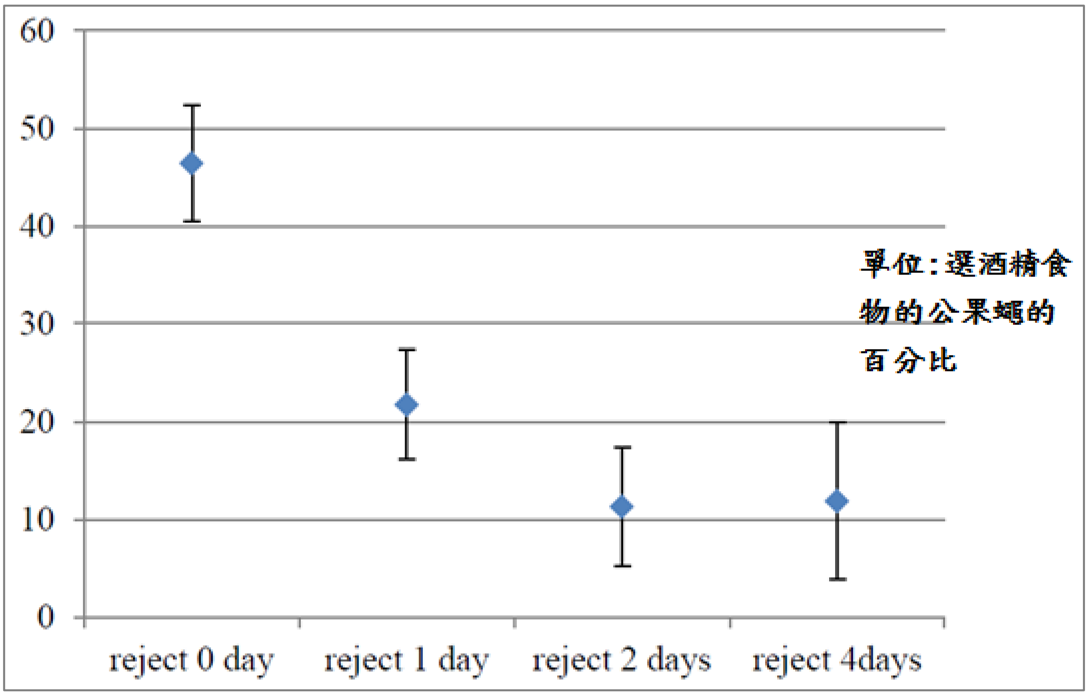


Fig.33，不同的拒絕程度，會造成不同程度行為改變嗎?

一、問題與討論:

(一) 數據分析與討論

1. 下圖為根據 Science 期刊中的實驗流程而繪出的流程圖，我們可以把整個流程分為二個階段。階段一為公果蠅求偶被拒後至其攝食第一次酒精，而階段二為攝食第一次酒精後直到測驗結束。透過實驗五「**公果蠅在被拒絕之後，是否是直接想去食用酒精？**」，我們發現被公果蠅在被拒絕後**(階段一)**並不會提升對酒精的趨向，而是透過攝食酒精後腦中的回饋學習而造成**(階段二)**。

2. 經過「**求偶動機實驗**」以及「**趨向實驗**」後，我們認為被拒絕的公果蠅的大腦一開始下的指令是「迴避酒精」以及 「積極交配」這兩個指令，而在食用酒精後被影響。而「迴避酒精」的原因，我們認為是因為公果蠅被拒絕後，攝食酒精將會造成其個體競爭力下降，因為從求偶實驗可看出，食用酒精的公果蠅會更為消極不去追求母果蠅。導致公果蠅在被拒絕時，大腦會下達「迴避酒精」的指令以減少其吃到酒精的機率。然而在食用酒精後，公果蠅會增加酒精的攝食量，我們從乙醚實驗的結果推測，是因為酒精與乙醚共有的「非選擇性神經鎮定劑」功能，阻斷了「NPF 下降」的信號，因此才會讓「迴避酒精」的指令失效，而為什麼失效反而會“提升”而不是與成功交配果蠅“打平”對酒精的攝食量，我們會在後面討論。

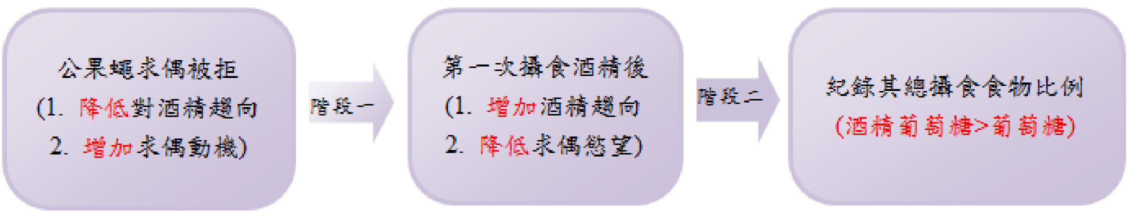


Fig.34 第一階段之實驗結果

3. 從階段三的實驗可看出，NPF 神經活性的變化的確會改變腦中 NPF 的量，並改變公果蠅的行為(1. 求偶行為 2. 酒精攝食行為 3. 對酒精之趨向)，但卻不會與被拒絕果蠅完全相符。第一種可能性是公果蠅在被拒絕的情況下雖然會減少 NPF 在腦中的量，但是減少的量卻不會與 NPF-Gal4+UAS-kir2.1 一樣多，mate 組與 NPF-Gal4+UAS-dTrpA1 亦然，也就是說其行為的不同可能是因為不同 NPF 的量所造成。第二種可能是除了 NPF 之外，公果蠅腦中還有其他神經傳導物在影響公果蠅之後的行為。這兩種可能性可以同時存在，我們認為同時存在的可能性最大。以下的圖示為此階段神經調控的流程圖:

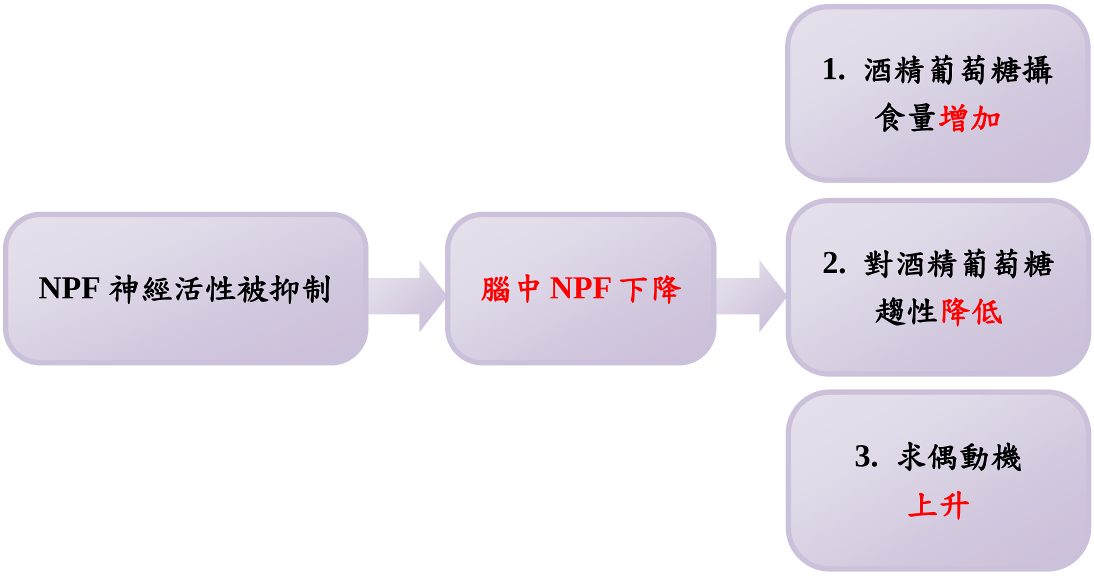


Fig.35 NPF 神經活性改變以及 NPF 所調控果蠅行為

4. 被拒絕的公果蠅的行為改變是否符合情緒行為的定義?

根據我們的實驗結果，我們發現公果蠅在食用酒精後，會阻斷 NPF 信號，進而提升酒精攝取量。然而阻斷信號理論上只會讓被拒絕的公果蠅攝食量恢復與一般果蠅相同，並不會刻意增加飲食。我們懷疑有兩種可能:一、NPF 能讓果蠅產生類似人類的負面情緒，而當公果蠅食用酒精，會使原本的負面情緒因為酒精的麻醉效果而改善，因此公果蠅會如同成

癮般不停食用酒精以維持「愉悅的心情」。另一種假設是，酒精和乙醚其實並不是阻斷神經的傳導,而是促使產生另一種傳導物可以與NPF拮抗，使大腦產生「增加酒精攝取」的指令。

我們認為前者的可能性較大，為了證明這個論點，我們查了許多文獻，想要了解科學上對情緒行為嚴謹的定義。最後找到了一篇文章“Two Different Forms of Arousal in Drosophila Are Oppositely Regulated by the Dopamine D1 Receptor Ortholog DopR via Distinct Neural Circuits”，本文探討了要構成情緒所需要具備的三個要素:

(1) 反應能夠持續(persistent)

(2) 刺激有程度之分(graduation of intensity)

(3) 對刺激有正面或負面的傾向(positive and negative reactions)

只要符合這三項條件的行為，就會被稱為類情緒驅動行為(emotion-like behavior)」。而階段四便是在驗證被拒絕的公果蠅的行為改變符不符合類情緒行為所必須擁有的三個條件。

而最後一項條件 valence(正趨性或負趨性)只是用於決定該刺激對生物體產生的是正面或負面情緒。由這個研究結果指出 NPF 可能是除了多巴胺(dopamine)外，另一種可以調控果蠅情緒的神經傳導物質。

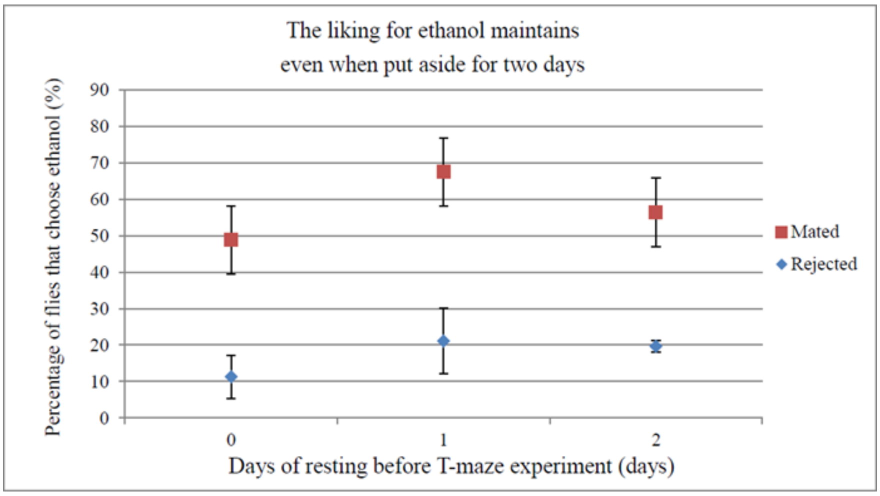
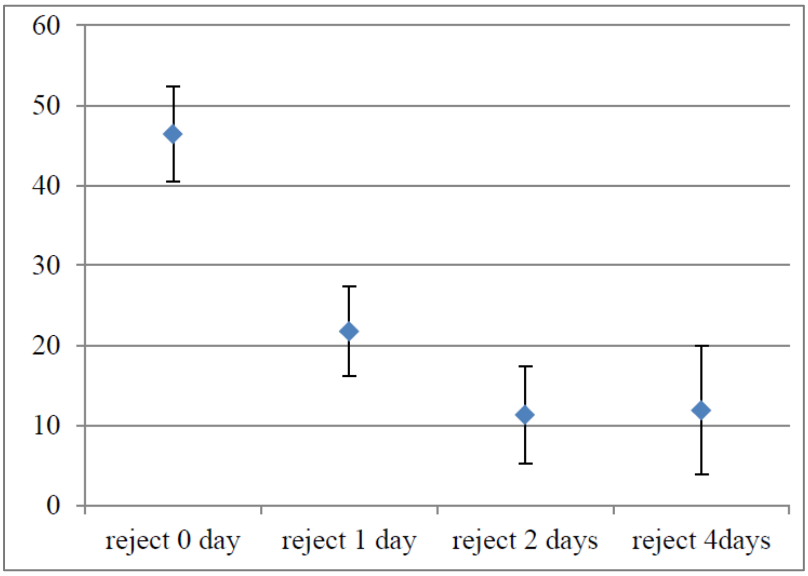


Fig. 36 左圖:公果蠅被拒絕後行為改變是否有持續性?
我們發現被拒絕的公果蠅在拒絕的流程結束後，放置一天、兩天，其反應仍會持續，符合 persistent 這個條件。

Fig. 37 右圖:不同拒絕程度，會造成不同程度行為改變嗎?
我們發現，隨著被拒絕的天數增加，公果蠅的行為改變也會跟著增加，此實驗結果符合條件二 graduation of intensity。



肆、　結論與應用

目前已經可以確定被拒絕之公果蠅提升對酒精的攝取量的原因並非是「大腦在被拒絕之後，直接下達提升酒精攝取量的指令」，而是被拒絕之後，酒精在腦中影響神經傳導物 NPF 所傳出之訊號，形成像麻醉了負面情緒一般的作用，使得被拒絕的公果蠅對酒精上癮。除此之外，公果蠅在被拒絕後，求偶的欲望會明顯增加。

而根據階段三的實驗我們可以建構出以下的調控流程圖:

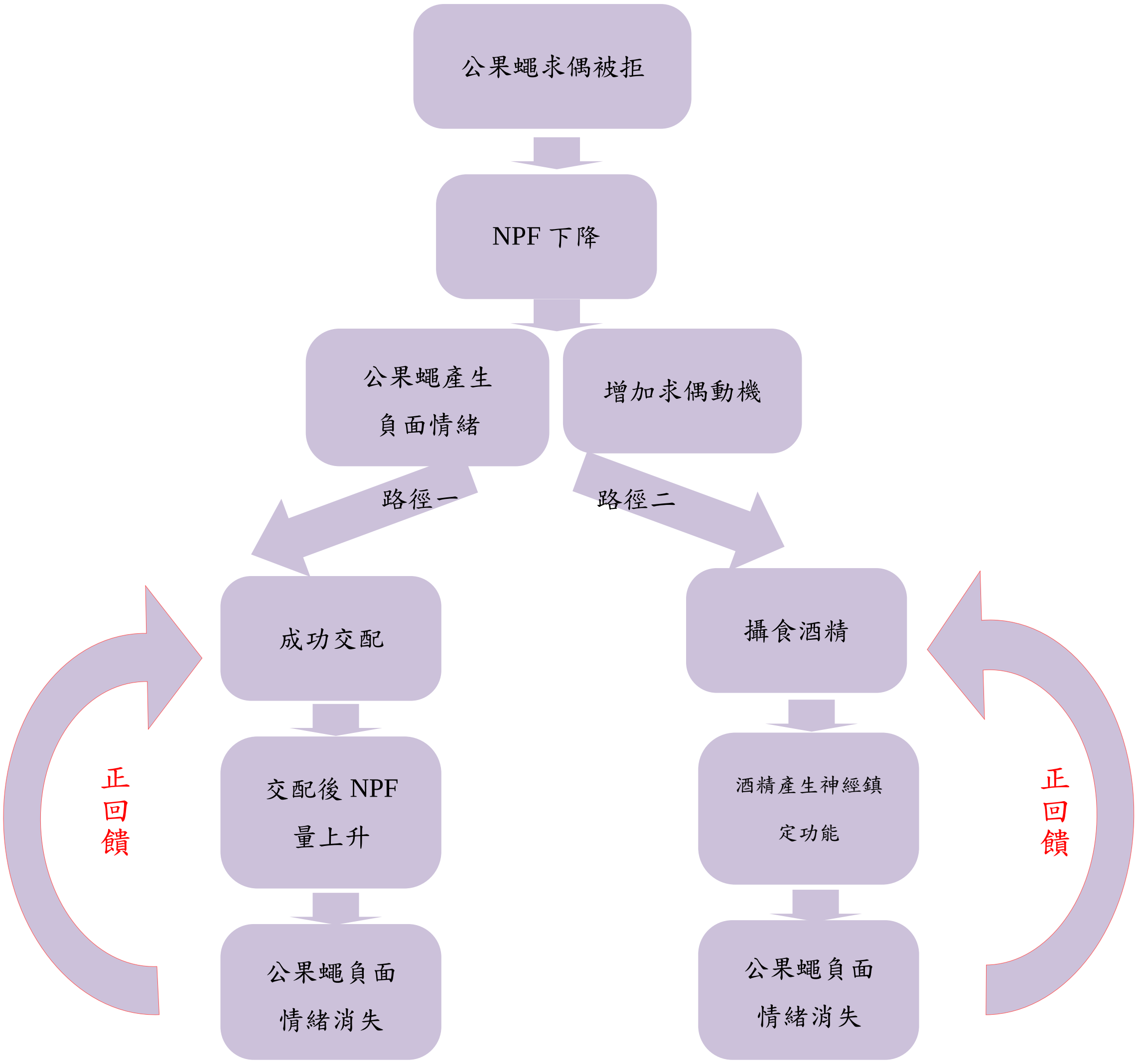


Fig.30 公果蠅被拒絕後，NPF 調控果蠅行為之路徑圖

而經由階段四之實驗可以得知，求偶被拒的公果蠅產生的一連串行為改變，是符合科學上「類情緒行為」的定義，而這個結果指出:NPF 很有可能是除了 dopamine 外另外一種可以控制果蠅情緒的神經傳導物質。

最後根據階段二至階段四的實驗結果，我們建構出公果蠅在被拒絕後，其行為改變的可能調節路徑:

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I. Introduction

Motivation

At 2012, a research on *Science* stated that if male fruit flies are rejected when they court females; they increase the intake of alcoholic foods. It caught our interests and our mind starts storming with questions. Do fruit flies have feeling? Do they really go for a "glass of beer" when they are "dumped"? Alcohol plays a complicated role in the Nature, it's a food source for many organisms and it's also a non-selective depressant. Thus, we wanted to find out what role does alcohol play in this behavior.

Another point mentioned in the article is that when male fruit flies are rejected by females, the amount of Neuropeptide F decreases. NPF is a homolog of Neuropeptide Y, a neurotransmitter already known to affect mammal feeding behaviors and emotions. Hopefully, we can figure out the relationship and extend our results to other species, for example, humans.

Background

1. Fruit Flies (*Drosophila melanogaster*)

Fruit flies are ideal lab animals. It has plenty advantages such as small size, short life cycle, easy to raise and it only has four pairs of chromosomes, a tiny number compared to us humans. Fruit flies are also similar to

Fig.1 Male fly (bottom) courting

humans in memory, senses and learning. Fruit flies share up to seventy percent of brain diseases we humans have, making it a perfect tool to genetic and behavior experiments

Courtship behaviors of fruit flies include chasing, contact, wing vibrating and various movements. When female flies start laying eggs, it refuses to further mate with other males. Using such behavior, we can control whether the male flies get rejected or not.

2. GAL4-UAS system

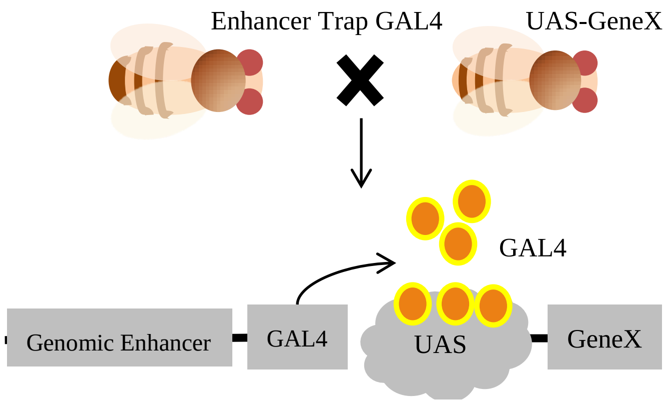


Fig.2 The function of GAL4-UAS system

To understand the genetic functions of fruit flies, scientists often use different gene tools to achieve their goal. Among them the GAL4-UAS system is quite frequently used. GAL4 is a transcription factor from yeast and is inserted into the fly's chromosome. The GAL4 expression timing and pattern can be regulated by the adjacent enhancer/promoter element. GAL4 will bind to the Upstream Activation Sequence (UAS), a transcription factor binding site, which only responds to GAL4, acting like a key and lock. The GAL4-UAS binding will activate the expression of the gene fused to UAS. We can further control the express of different genes in specific tissues by using different combinations of GAL4/UAS-transgenes. By binding

NPF-Gal4 with UAS-kir2.1 it activates the potassium channels of NPF neurons so it can’t depolarize, further decreasing its activity (Baines et al., 2001;Paradis et al., 2001). While bonding with UAS TrpA1 decreases potassium activity and enable cells to polarize, resulting in increases neuron activities (Rosenzweig et al., 2005;Rosenzweig et al., 2008).

II. Experimental Methods

Instruments and Materials

Materials:

Fruit flies (*Drosophila melanogaster*)	glucose solution	ethanol	ether	fly food

Instruments:

dissecting microscope	capillary (75mmx1.15mm)	culturing tubes	
glass jars	confocal fluorescence microscope	T-maze	forceps


Fig.3 Drosophila culturing tubes10*10


Fig.4 Culturing glass jars


Fig.5 Dissecting microscope


Fig.6 T-maze

Fig.7 Confocal fluorescence microscope

Fig.8 Tools for brain dissecting and slide making

The main Objectives of our experiment

1. To find out which concentration of the ethanol/ether glucose is favorable and repeat the experiment on the Science Journal. (Stage 1 experiment)
2. To explain why the rejected male flies increase ethanol intake. (Stage 2 experiment)
3. To define what behaviors the NPF controls. (Stage 3 experiment)
4. To figure out whether '' emotion is involved'' or not. (Stage 4 experiment)

Raising process of the males

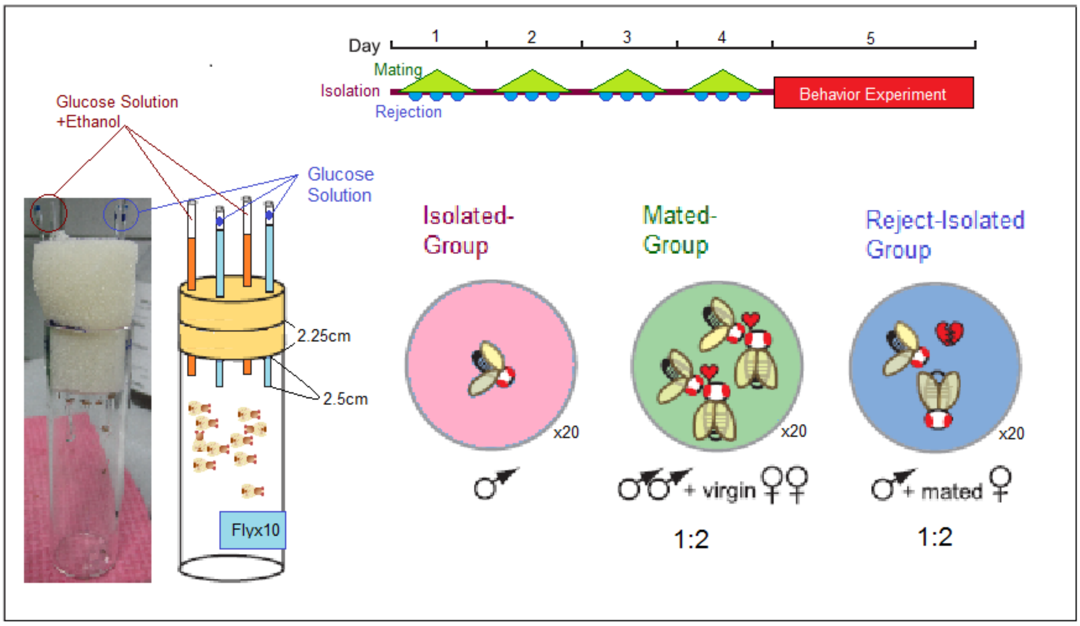


Fig.7 Raising protocol for different groups of flies

There are three groups of males used in our experiment:

1. Isolated-group: The flies in isolated group are raised individually and acts as wild type in further experiments.

2. Mated-group: The Mated group is to mate six hours with females once per day, with the arrangement of two females versus one male.

3. Rejected group: The Rejected group is scheduled to court female flies that are laying eggs. So the males will be rejected by these mated females. We place the male flies with the samenumber of females as the mated group.

These three groups are often used in our further experiments.

Experimental methods

Stage 1: Ethanol/Ether concentration and the repeat of the experiment on Science

(1) The trend of fruit flies toward normal food and ethanol-containing food

(2) The trend of fruit flies toward normal food and ether-containing food

(3) Ethanol food **consumption** of the rejected males

Stage 2 : Possible function of the ethanol in rejected male flies

(4) Ethanol **preference** of the rejected male with or without ethanol intake

(5) Mating motivation of the rejected males

(6) **Ether** food consumption of the rejected males

Stage 3 : Behaviors controlled by the NPF

(7) Ethanol food consumption of the NPF-lacking males

(8) Ethanol preference of NPF-lacking males

(9) Mating motivation of the NPF-lacking males

(10) The amount of the NPF in each male group

Stage 4 : Emotion-related experiments

(11) Persistent reaction of the males after being rejected

(12) Graduate reaction of the males after being rejected

Stage 1 : Preparation experiments

1. The trend of fruit flies toward normal food and ethanol-containing food
2. The trend of fruit flies toward normal food and ether-containing food
3. Measure ethanol intake of rejected flies
4. Climbing experiment for ethanol-fed flies

Stage 2 : Ethanol-related experiments

5. Fruit flies' liking towards ethanol
6. Motivation experiment of flies during courting
7. Measure ether intake of rejected flies

Stage 3 : NPF-related experiments

8. Measure ethanol intake of NPF-controlled flies
9. The instincts of NPF-controlled flies toward ethanol
10. Motivation experiment of NPF-controlled flies during courting
11. Location of NPF neurons in a fly's brain
12. Immunostaining of NPF

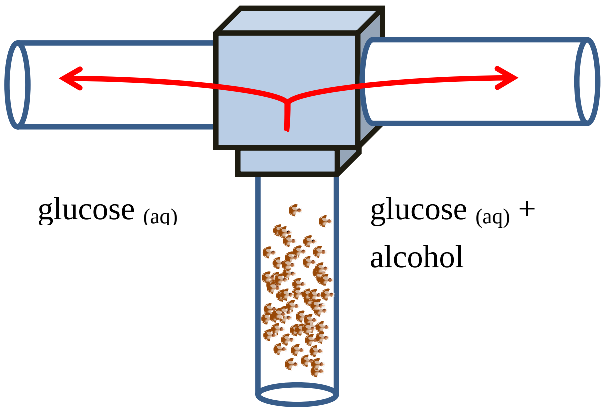
Stage 4 : Emotion-related experiments

13. Measure continuous behavior of rejected flies
14. The difference between different time length of rejection

Stage 1 : Preparation experiments

1. Is it appropriate to use 15% of ethanol?

In the papers we've searched, they say that 5~15% of ethanol is most ideal for ethanol related experiments. To assure that it's correct, we used the T-maze we designed to measure the fly's trend towards different concentrations of ethanol.



We first place glucose solution on the left side of the T-maze, and ones mixed with ethanol on the right, then we place 100 flies on the bottom tube. After three minutes, we counted the flies on both sides to know which food they like better. We repeated the same process once more with the foods placed on different sides. The concentrations of ethanol we would be trying would be from zero to a hundred percent, each group increasing by ten percent, also adding fifteen percent mentioned in the papers.

2. Is using 15% of ether also appropriate?

We also tried to replace ethanol with ether, but fruit flies might not prefer the same concentration of ether as ethanol. So to be sure, we repeated the same process above, but mixed the glucose solution with ether instead.

3. Do male fruit flies really increase ethanol intake when rejected by females?

To assure that the experiment is repeatable; we followed the steps on the article and repeated the experiment. We did some adjustments such as adding an isolated group and set the experiment time to twelve hours. First we raised the three groups of flies following to the protocol mentioned before. We then split the twenty flies of each group into to empty culturing tubes. We place the six tubes with other five empty control group in a shaded area for twelve hours. After letting the flies starve for an hour, we than replace the sponge on the tube with ones having four capillaries in them, two containing glucose solution and the other two with ethanol mixed within. After twelve hours, we take the capillaries out and measure the weight loss, then transfer it from grams to milliliters.

Stage 2 : Ethanol-related experiments

4. How do fruit flies act towards ethanol?

It's actually common for fruit flies to eat ethanol-containing food, for example, over-ripe fruits. So do rejected flies increase the intake of ethanol because they want to "get a drink", or do they just want to eat more food? To find out, we did the following experiment. We raise the three groups of flies 200 hundred each, and then run the T-maze experiment on them. By comparing the difference between the groups, we may know if there are changes of behaviors in rejected flies.

To our surprise, rejected flies avoid ethanol at first contact; acting differently from the twelve-hour feeding.so we did another experiment. First we raise two hundred flies for each group, and then divide them into two subgroups. The first three groups we ran the T-maze experiment, with ethanol and normal food on each side, on them after two hours of starvation. Then we counted the number of flies on each side after three minutes. The other groups, we fed them both normal food and ethanol food for thirty minutes each after the two-hour starvation. Then we run the T-maze experiment on them after another two hours of starvation.

5. **Are there other behaviors that changed when flies get rejected?**

We were thinking "What if the food choice isn't the only thing that changed when flies get rejected?" We imagine that if fruit flies "drink alcohol" like humans, they might act like us, too. Therefore, we designed an experiment on the courting behavior of the flies. First we raise four groups of flies following to the chart below.

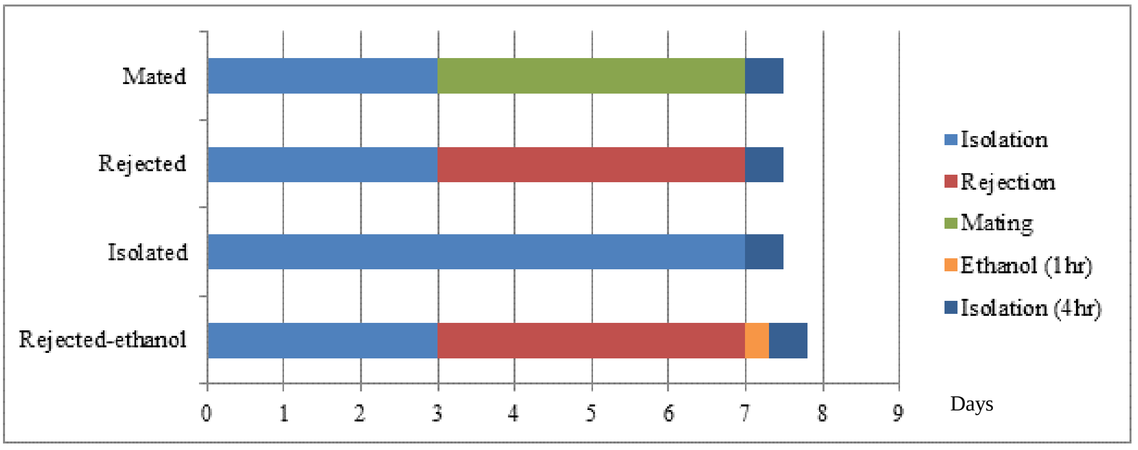


Fig9. Protocol for raising the flies in the courting experiment

1. Mated: same as the mated group in ethanol experiment

2. Rejected: same as the rejected group in ethanol experiment

3. Rejected-ethanol: This group followed the same protocol as the rejected group, but we fed them an hour of ethanol food before isolation.

After raising them for four days, we isolate them for four hours before placing them with females. Then we recorded the time need for them to start courting female flies.

6. Do male flies also increase the intake of ether after being rejected?

We do not know what kind of role does alcohol play in this behavior, and there are all kinds of possible conclusions. Ethanol might help by blocking the neuron signals to make the flies feel “better”. Yet it also might be the enhancer of a brand new reaction that fights with the current one. We took a leap in the dark and assumed that ethanol might acts as a depressant for neurons. We replace ethanol with ether, and then reduced the concentration to 10 % according to our preparation experiments. If the results of the ether experiment are similar to the ethanol experiment, we may conclude that ethanol does work as a depressant in the flies’ brains.

Stage 3 : NPF-related experiments

Background

In stage 3, we further extend the experiment to the relation with NPF. Other than the original three groups of flies, we added five more using the GAL4-UAS system to control the activity of NPF neurons. The only difference about the ways we raise them, is that they are raised at the temperature of 30℃ in order to trigger the UAS within them.

1. NPF-Gal4+UAS-kir2.1

Disruption of membrane depolarization is a way to silence neurons. Neurons open voltage-gated sodium channels in response to membrane depolarization to propagate action potentials or graded changes. UAS-kir2.1 encodes a mammalian inward rectifying K^+ channel and its expression provides the most complete suppression of depolarization of the reagents. The UAS-kir2.1 we used are recombined with other tools to make it temperature sensitive allowing us to control when to activate it. By raising the temperature above 28℃ we can lessen the activity of NPF neurons.

2. NPF-Gal4+UAS-dTrpA1

Neurons can be rendered more active by increasing sodium or calcium conductance, or by reducing potassium conductance. Opposite from UAS-kir2.1, the temperature activated cation channel UAS-dTrpA1 has been a powerful reagent to acutely activate neural activity and has been used to identify neurons involved in sleep and courtship behavior. The acute activation in response to moderate temperature increase and the sustained depolarization have made UAS-dTrpA1 a favorite tool in many labs. The temperature for UAS-dTrpA1 to active is also at the temperature of 28℃. Therefore we raised the flies at the temperature of 30℃, allowing both reagents to activate.

3. NPF-Gal4+ w1118

4. UAS-dTrpA1+w1118

5. UAS-kir2.1+w1118

The three groups above acts as control group to the first for NPF-Kir2.1 and NPF-dTrpA1. To prove that any difference in behavior is caused by the combination of Gal4 and UAS, not by one alone. If the change of behavior is caused by NPF, supposedly the three groups should act similarly to the isolated group.

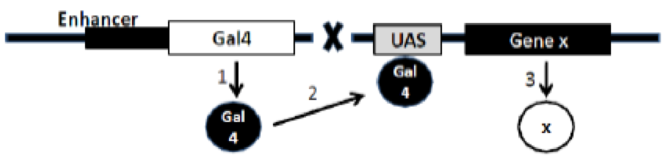


Fig.10: To control the activity of the NPF neurons, we use the NPF-Gal4+UAS-kir2.1 which can produce protein X to increase the potassium channel's activity of the NPF neurons, making the NPF neurons hard to be activated.

Experiments

At this stage of experiment, we take the eight groups of fruit flies and run three experiments on them:

7. **Ethanol consumption experiment (same process as the third experiment)**
8. **Ethanol preference experiment (same process as the foruth experiment)**
9. **Mating motivation experiment (same process as the fifth experiment)**

By comparing the results with the results of stage 2, we then can know whether NPF does or doesn't affect the behavior of rejected flies.

10. We then tried immunostaining, which stains the NPF itself, allowing us to know the current amount of NPF in the fly's brain. The flies we did immunostaining include rejected, isolated, mated flies and NPF-Gal4+UAS-kir2.1, NPF-Gal4+UAS-TrpA1 flies.

Stage 4 : Emotion-related experiments

The fact that rejected male flies increase the consumption of ethanol after trying it caused a questioned stirring in our mind. “Is it possible that flies have emotions?” After reading an article called “Two Different Forms of Arousal in Drosophila Are Oppositely Regulated by the Dopamine D1 Receptor Ortholog DopR via Distinct Neural Circuits” they defined that “emotion-like behavior” should include three conditions:

1. Persistent
2. Graduation of intensity
3. Positive and negative reactions

It is these three conditions distinguish the difference between emotional-like behaviors and simple reflexes. So we designed the following experiments to figure out whether the actions changed after being rejected fits the three conditions of emotion-like behavior.

11. Is the behavior of rejected flies persistent?

We let the rejected flies “cool down” for one to three days, then re-run the T-maze experiment on them. Then observe if there are any differences while the “cool down” stretches out.

12. Do flies act differently if they were rejected for other periods of time?

We split the flies into three groups. Letting them get rejected for one, two , and four days. Then we run the T-maze experiment on them, and see if their disliking for ethanol becomes more intense when rejected for a longer period of time.

III. Results

Stage 1 : Preparation experiments

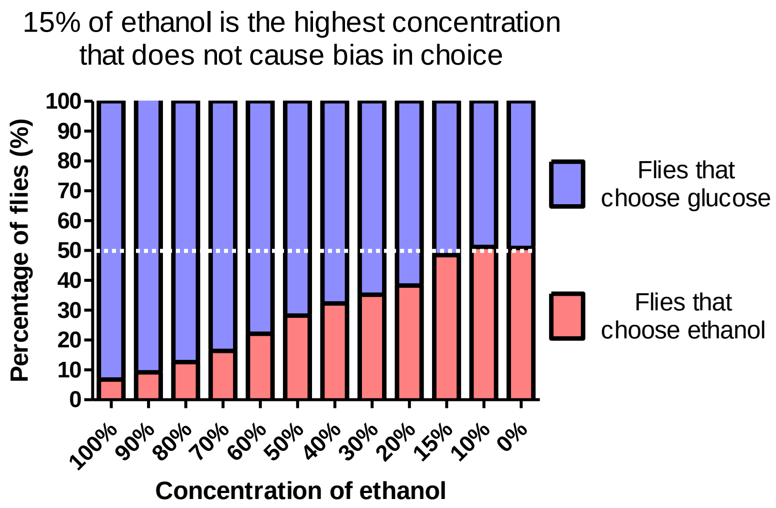


Fig.11 The trend of fruit flies toward glucose solution and ethanol-containing solution

1. First we need to find out if it is true that flies prefer ethanol concentrations around 5~15%. We place normal flies into the T-maze and recorded the flies on either side of the tube, while decreasing the concentration by ten from 100 to 0.

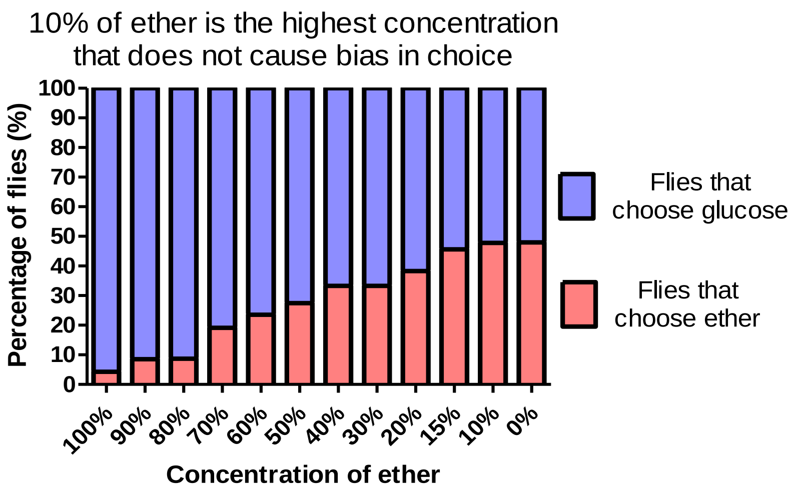


Fig.12 The trend of fruit flies toward glucose solution and ether-containing solution

In Fig. 12 we can see that fruit flies avoid food with high concentrated ethanol. At the point between 10~15%, the ratio of the flies on both sides are closest to 1:1. Hence it is appropriate using 15% of ethanol in experiments. The reason we chose 15% instead of 10% is to increase the difference as much as possible, yet still within acceptable range.

2. We used ether instead of ethanol in several experiments to check if flies accept the same concentration of ether as ethanol. Therefore, we repeated the same experiment above but replaced ethanol with ether.

We can see in Fig.12 that the chart for ether concentration is highly similar to the ethanol concentration. However, we decided to use 10% of ether to run experiments. The reason is though it seems that flies accept 15% of ether, their body can't withstand the chemical effect and faints before consuming it. If this happens, we fail to know whether the rejected flies really prefer ether over glucose, or they simply don't have the chance to refuse since they fainted right away.

3. To further extend our experiments, we need to first assure that the experiment mentioned in the article is repeatable. We followed the same steps mentioned and repeated the feeding experiment.

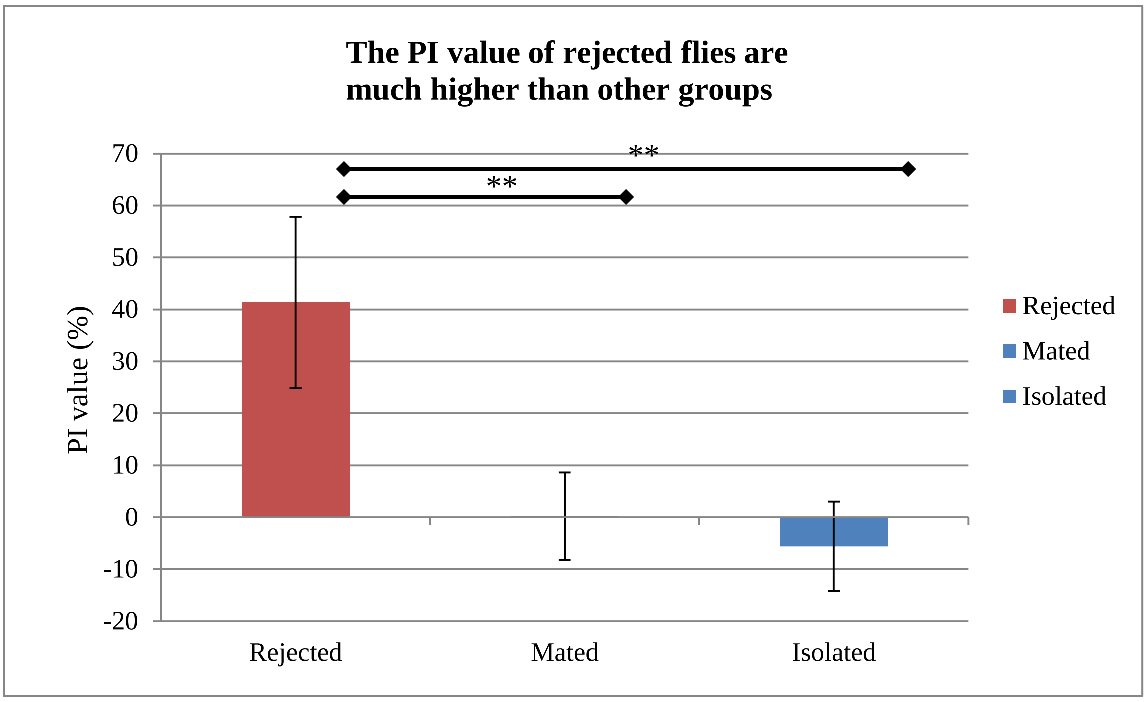


Fig.13 The PI value of the flies' consumption
PI= (ethanol intake − glucose intake)/ total intake x 100%

The PI value in Fig.13 is defined as the percentage of ethanol consumption subtracting the percentage of glucose. The 40% of the rejected group means 70% of ethanol food intake minus 30% of normal food.

The result shows that rejected flies prefer eating ethanol food rather than glucose. The isolated group acts similarly to the mated group, meaning that the isolation during fly-raising is not the main reason causing the change of behaviors.

Stage 1 Summary

After testing the flies' liking toward ethanol and ether, we decided to use 15% of ethanol and 10% of ether in our future experiments. We choose the maximum concentrations for both solutions that would not cause bias in the flies liking, so if there was a change in their choice, it would cause a larger difference then it will in lower concentrations. We were also certain that the feeding experiment was repeatable and did not have dramatic changes when repeated. Finally we decided to let flies feed on ethanol for one hour and rest for four hours in future experiments based on results of the climbing experiment.

Stage 2: Ethanol-related experiments

4. We were curious about whether flies increase ethanol preference right away after being rejected. So we did an experiment using the T-maze. We separated the rejected group into two subgroups. In the first group, right after the four-day rejection, we put them in two the T-maze for three minutes to test their ethanol preference. In the second group, we forced them to consume ethanol food for one hour before putting them into the T-maze. (We also repeat the process above and toward.

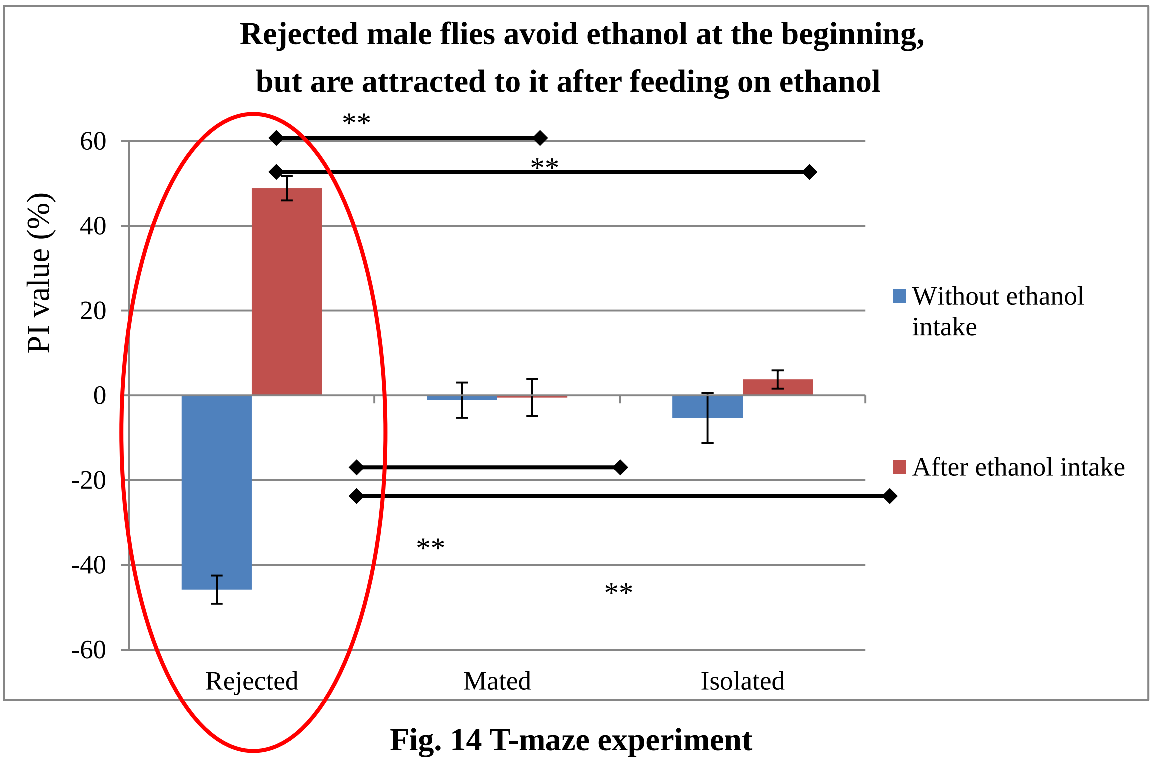


Fig. 14 T-maze experiment

The result showed that the males avoided ethanol food right after the four-day rejection. However, after we forced them to consume ethanol food, the rejected male's ethanol preference was reversed to stronger compared with other groups.

5. We wondered if it is possible that flies may act differently towards females when being rejected. We raised three groups of flies including rejected, mated, and rejected flies fed with ethanol. We fed the "Reject-ethanol group" ethanol for an hour and let them rest for four before letting them court with females.

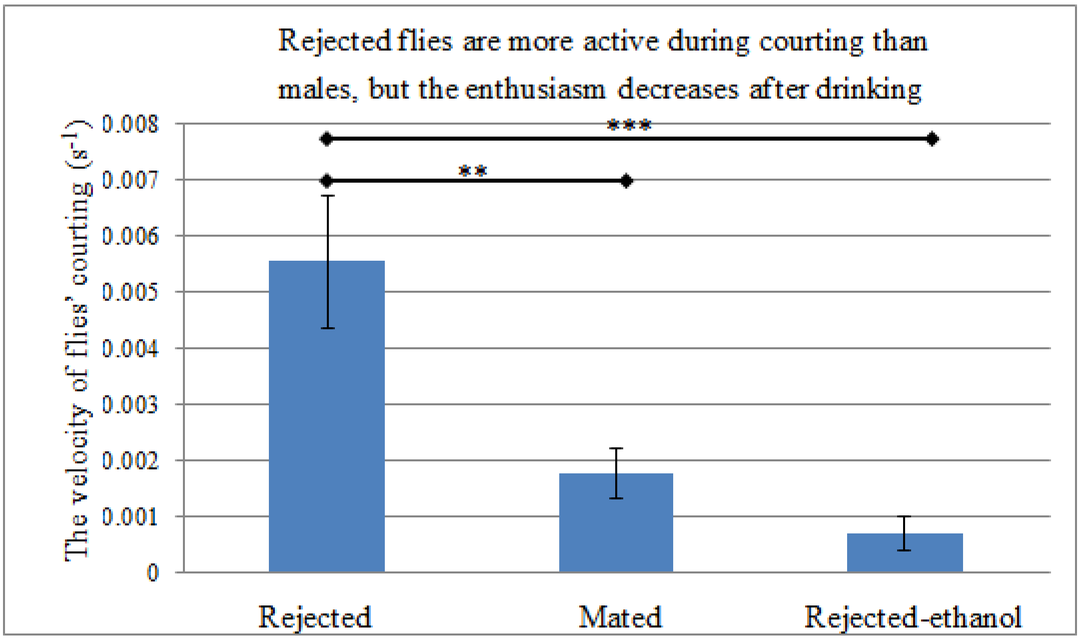


Fig.15 Time needed for flies to court females

We see in Fig.15 that rejected flies are more active than mated flies when it comes to courting. But if the flies happen to eat ethanol food before courting, its enthusiasm decreases dramatically.

6. We supposed that ethanol have two possible roles in the nature. One acts as a food source for many organisms, another is a depressant that blocks neurons signals. To find out, we repeated the twelve-hour feeding experiment but used ether, a depressant that organisms don't eat as food, instead.

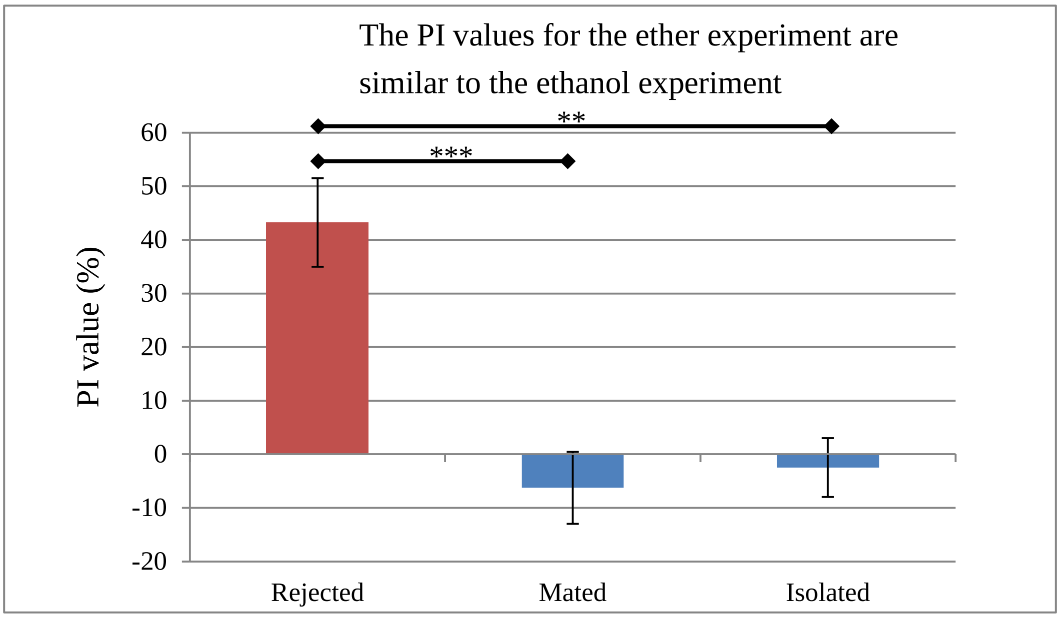


Fig.16 The PI value of the flies' consumption

According to Fig.16, we can see that flies have a similar liking for ether and ethanol.

Stage 2 Summary

1. Ethanol food consumption response after rejection can be separated into two stages. Right after the four-day rejection, the male flies actually avoid ethanol. However, after ethanol consumption, their ethanol avoidance becomes reversed to ethanol preference that is stronger than normal.

2. The function of the ethanol might be signal blocking.

3. The rejected males will increase their courting motivation after being rejected.

Stage 3: NPF-related experiments

7. In the article it mentioned that the amount of NPF decreased in the brains of rejected flies. So we wanted to find out if NPF control the ethanol consuming behavior in drosophila. We repeated the experimental process as the experiment.3 and added five groups to GAL4-UAS segments in them. Among the five, **NPF-GAL4+UAS-Kir2.1** suppressed the activity of NPF neurons, and **NPF-GAL4 +UAS-dTrPA1** did otherwise. The rest acted as control groups.

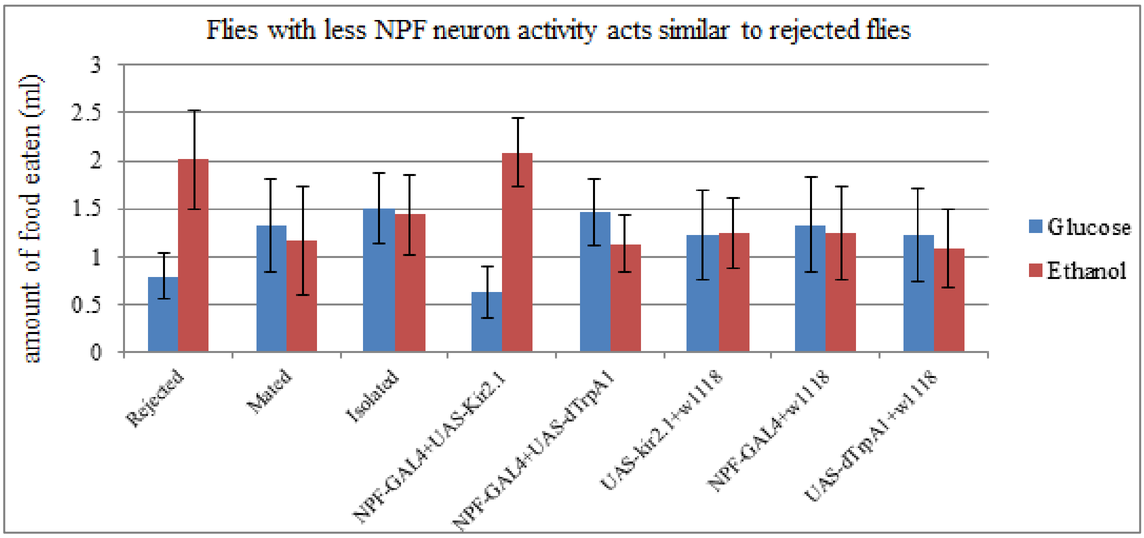


Fig.17 The consumption of ether and glucose

We can learn from Fig.17 that flies with lower NPF neuron activities act similarly to rejected flies. This means that the activity of NPF neurons can control male flies ethanol food consumption.

8. We can infer from the former experiment that flies with lower neuron activities may appear similar to rejected ones. Then, does the amount of NPF in brain affect the ethanol preference in drosophila? We took the eight groups of flies and ran the ethanol preference experiment for the first subgroup on them, recording the flies on both sides when placed into the tube after two-hour isolation.

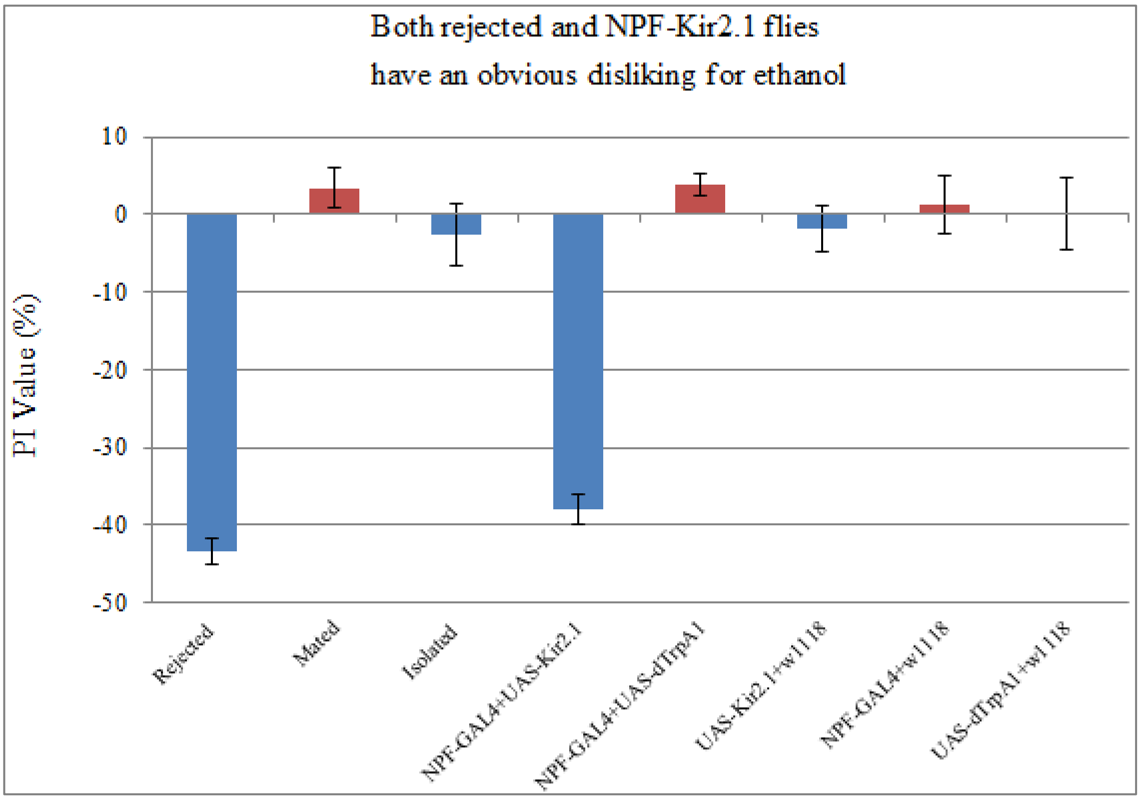


Fig. 18 T-maze experiment

From Fig.18 we can see that although NPF -Kir2.1 and rejected flies are not completely identical. Both groups decrease their ethanol preference before ethanol intake. And the increase of neuron activity (NPF- TrpA1) does not make a huge difference to the flies' behavior, just like mated flies.

9. We also tried the courting experiment on the GAL4-UAS flies.

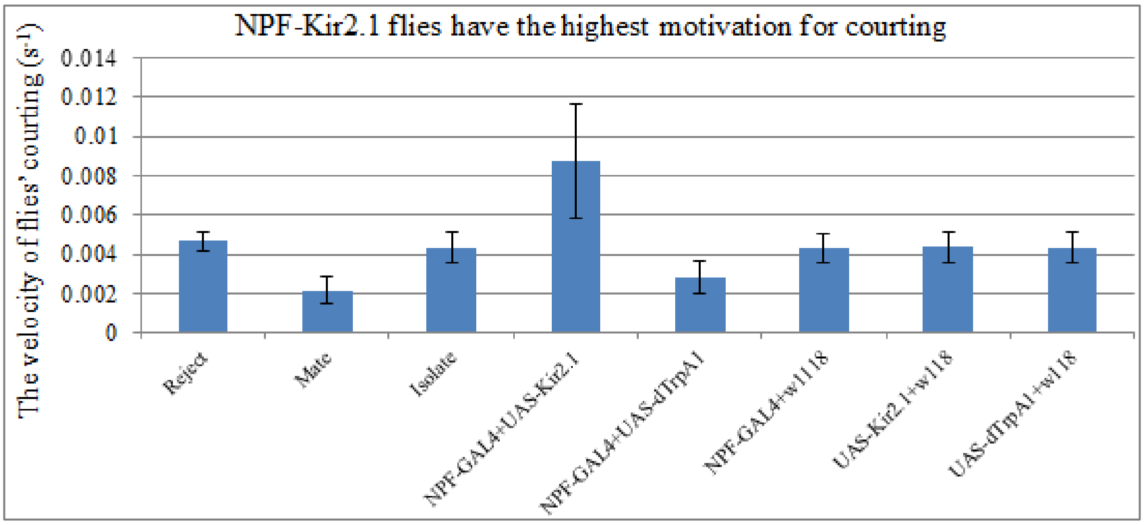


Fig.19 Courting experiment

From the chart (Fig.19) we can see that the motivation for NPF-Kir2.1 flies to court females is the strongest among the groups, and the results of NPF-TrpA1 flies are similar to mated flies. We can therefore conclude that the activity of NPF neurons can control the male's mating motivation.

10. In this experiment, we stained the NPF protein with antibodies with green fluorescence. Then scan the amount of NPF in the flies' brain in rejected, isolated, and mated flies.

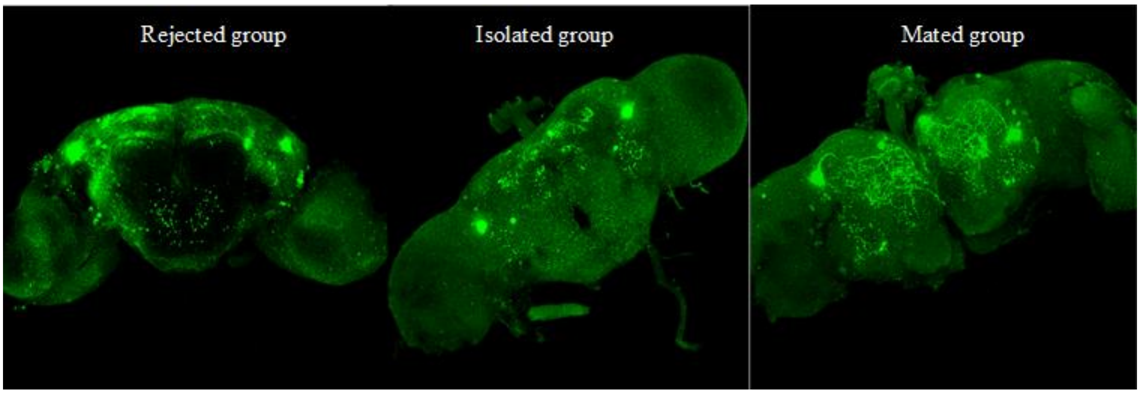


Fig.20 Locations of NPF in the brains of rejected, isolated, and mated flies

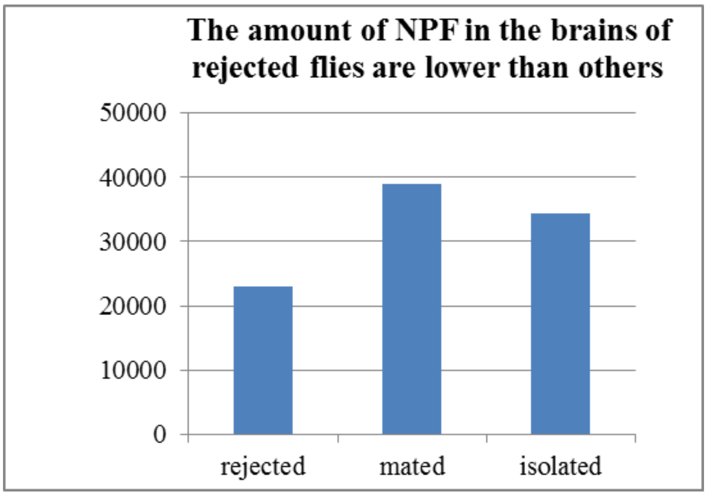


Fig. 21 The area of NPF proteins

In Fig. 21 we can see that the amount of NPF in mated flies nearly doubled the amount in rejected flies. So it is true that when Male flies are rejected by females, the amount of NPF in their brain does decrease.

We then stained the fly brains of NPF-Gal4+UAS-Kir2.1 and NPF-Gal4+UAS-dTrpa1 flies, to make sure that the activity of the NPF neurons do will influence the amount of the NPF.

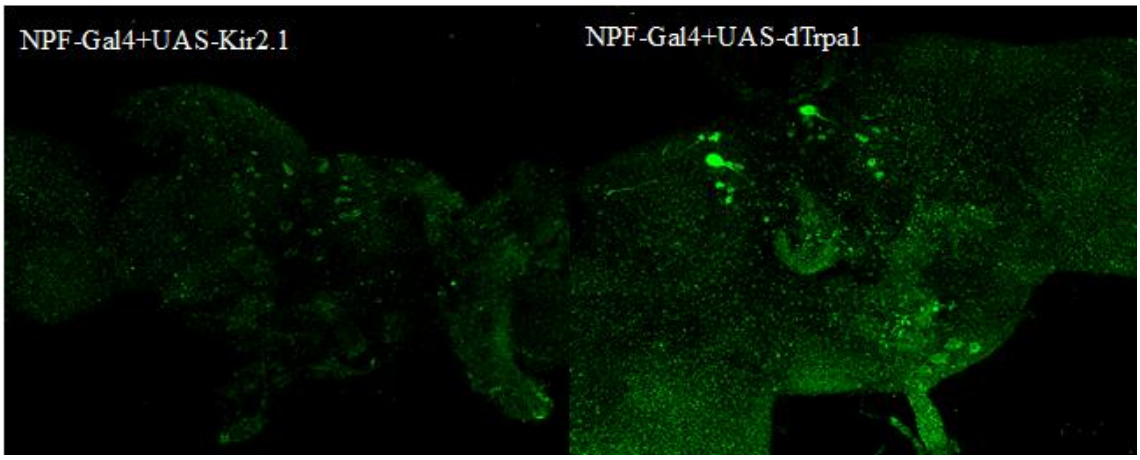


Fig.22 The locations of NPF in the brains of NPF-Gal4+UAS-Kir2.1 and NPF-Gal4+UAS-dTrpa1

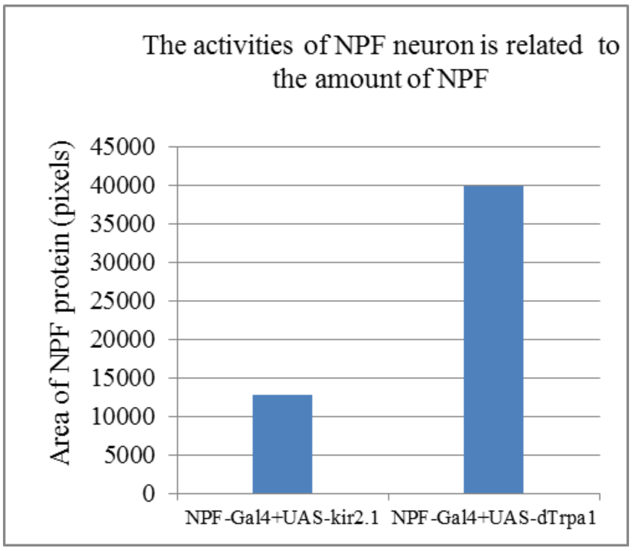


Fig.23 The area of NPF proteins

In Fig. 23 we see that the NPF-Gal4+UAS-Kir2.1 males have lower NPF amount compared with the NPF-Gal4+UAS-dTrpa1.

Stage 3 summary

In the experiments in stage four, we discovered that the lacking of NPF has similar effect on flies in mating motivation, ethanol food consumption, and ethanol preference as rejected flies.

Stage 4: emotion related experiments

After the experiments done in stage 1 to 3, we can't help but think whether it is possible for fruit flies to have feelings and emotions. After research, we found an article titled "Two Different Forms of Arousal in Drosophila Are Oppositely Regulated by the Dopamine D1 Receptor Ortholog DopR via Distinct Neural Circuits" talking about emotion-like behaviors of fruit flies. In it they defined that "emotion-like behaviors" contain three properties: 1. Persistent 2. Graduation of intensitity 3. Valence(positive or negative emotion state). Therefore, we designed the first and the second experiment based on the first and the second properties.

11. The first experiment is to see if the behavior is persistent. In the first group, we isolated the rejected from males after four-day rejection for zero to two days, and put them into the T-maze for three minutes to test their ethanol preference. In the second group, we isolated the mated males for zero to two days and tested their ethanol preference in three minutes.

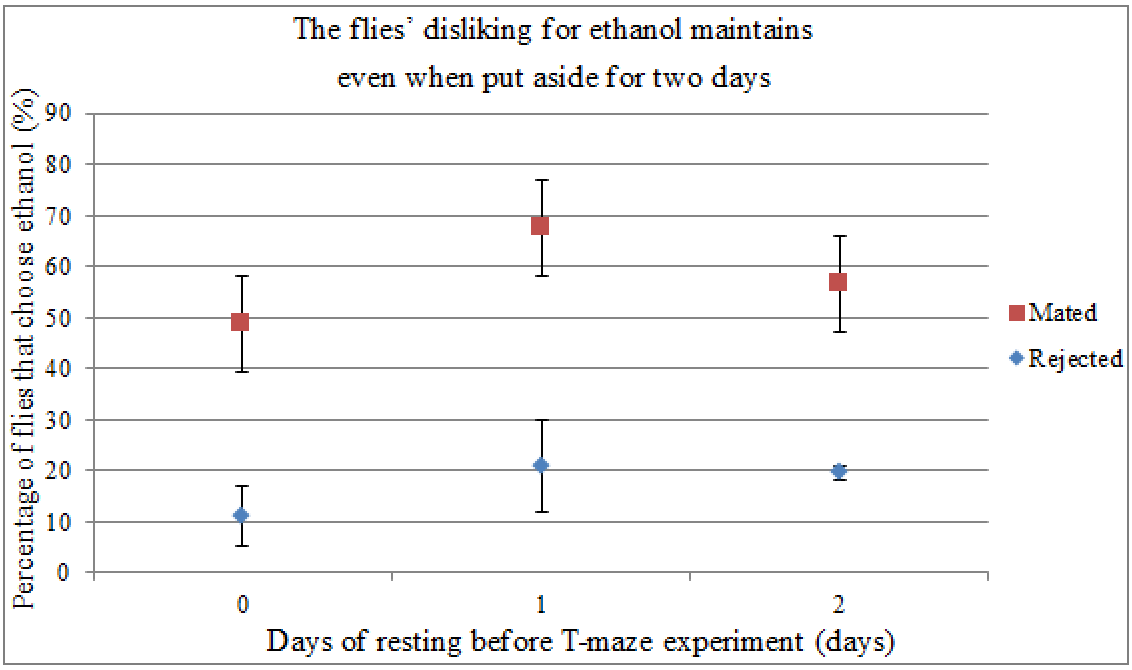


Fig.24 T-maze experiment on rejected flies that rested for different days

In Fig.24 we can see that the ethanol preference change in the rejected males can last for two days. According to the result, we can know that the behavior after being rejected is persistent.

12. The next condition is the graduation of intensity for the behavior. We split the flies into three groups, letting them get rejected for one, two, and four days. Then we ran the T-maze experiment on them, and saw if their disliking for ethanol would become more intense when rejected for a longer period of time.

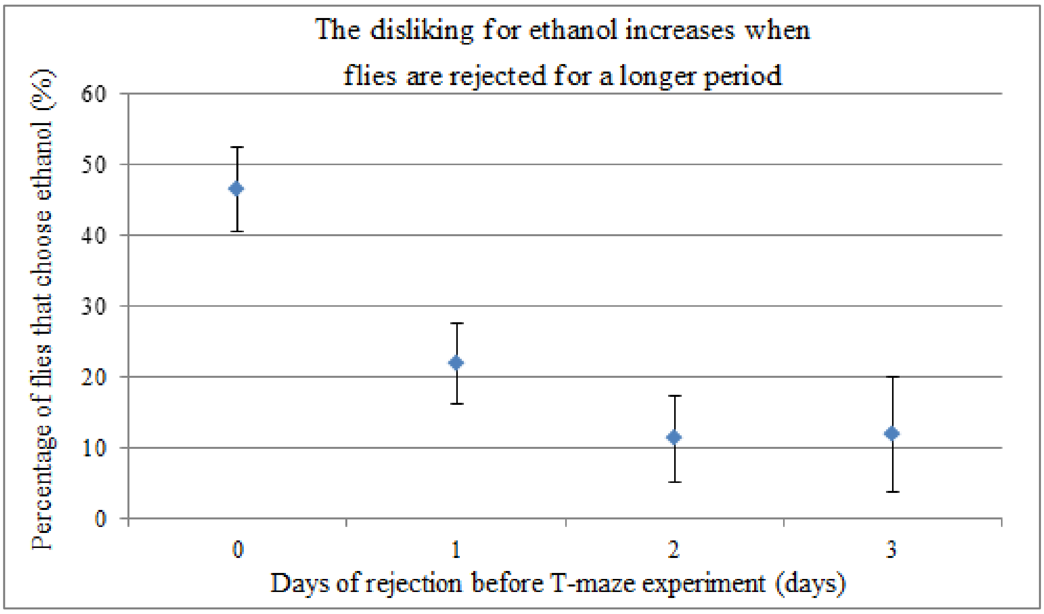


Fig.25 T-maze experiment on flies rejected for different days

We can see from Fig.25 that the more days the males got rejected, the more significantly their ethanol preference will change more significantly. So the behaviors after rejection do have a graduation of intensity.

Stage 4 Summary

After the two experiments, we can assume that the behaviors that occurr after being rejected are persistent and have different levels of intensity. And from the T-maze experiment we did in stage two; we can see that rejected flies have positive and negative preferences. Therefore we can conclude that those behaviors correspond to the ‘’emotion-like behavior’’**.**

IV. Discussion

Fig. 26 is a flow chart based on the article published on Science. And Fig. 27 is our new discovery compared with the path way Science article proposed.

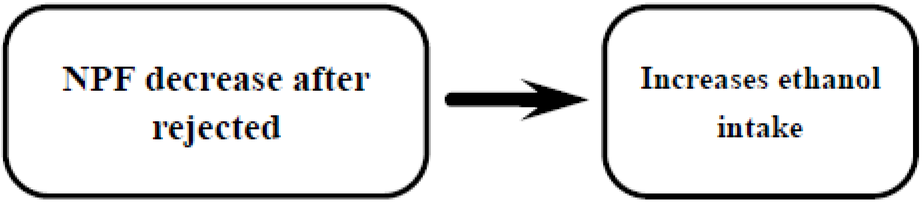


Fig. 26 Article stated that the NPF amount in flies decrease and the ethanol intake increases

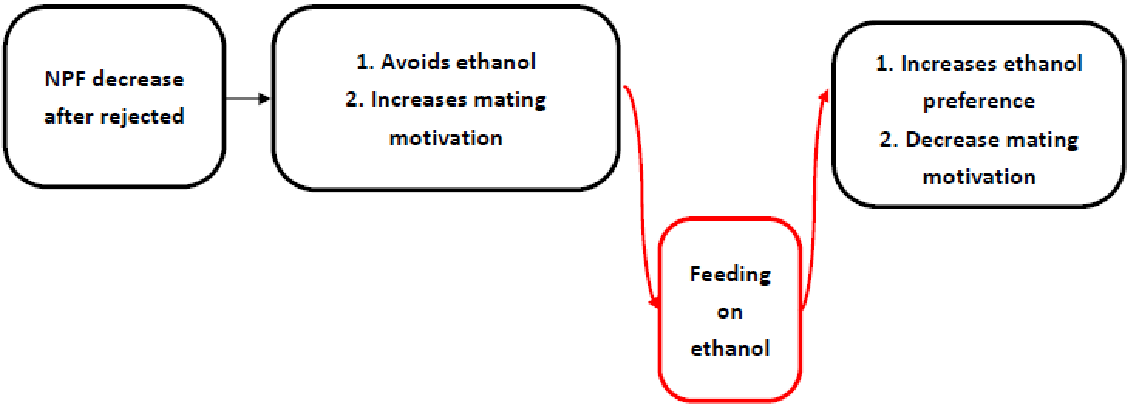


Fig.27 We discovered that flies have different reactions to ethanol in Stage 1 and 2

According to our results, we can roughly split the flies' behavior into two sections, before and after the first time they consume ethanol. And we can see that there is a dramatic change of behavior in the two sections. Flies decrease ethanol preference right after they get rejected, but increase ethanol preference after the ethanol intake.

But why do rejected flies avoid ethanol for the first contact? In our courting experiment, we can see that rejected flies feeding that had fed on ethanol court less than mated flies. So we made a assumption that it may be possible that the flies avoid ethanol to be more competitive, when the rejected males tend to increase their ethanol preference and decrease their mating motivation.

Supposedly alcohol acts as a depressant and blocks the signals of NPF, but that does not explain why their ethanol preference is reversed to stronger instead of returning to normal. It is possible that alcohol has more than one role in this behavior, and we will try different methods to find out in the future. For example, we may feed them other drugs instead of alcohol or try using dopamine to control their emotions.

In stage 4 we go through experiments to see whether the behavioral change in being rejected counts as emotion-like behavior. And the results showed that the reaction after the males got rejected do correspond to the ‘’emotion-like behavior’’. However, there are still two possibilities to explain why rejected male flies increase ethanol intake even when it might only block the NPF signals, returning their behavior to normal.

1. The lacking of NPF creates a signal similar to the bad feelings of humans in the males’ brain. After the male flies consumed the ethanol food, they learned that the ethanol could transfer their emotion state from negative to positive and tended to take more alcoholic food.

2. While of inhibiting the signal transmission of neurons, ethanol and ether also causes another reaction either creating another neuropeptide or stimulating production itself. It ends up with the same effect as the increasing of NPF and possibly drives the flies to consume more ethanol.

V. Conclusion

1. After the males get rejected (or the activity of the NPF neurons decreases), they will decrease their ethanol preference and increase their mating motivation.

2. If the males consume ethanol food after being rejected, they will increase their ethanol preference and decrease their courting motivation.

3. NPF can control male flies' ethanol food consumption, ethanol preference and mating motivation.

4. The reaction after the males got rejected corresponds to the ''emotion like behavior''.

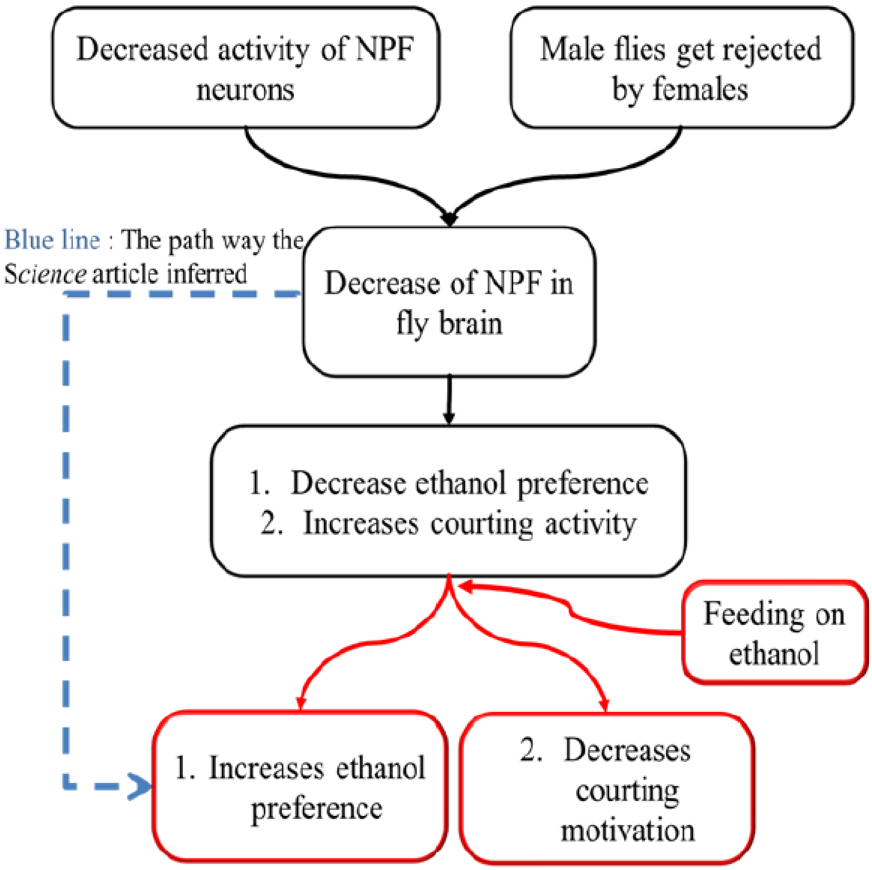


Fig.27 Newly discovery of present study compared with the data published on *Science*

VI. References

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評語

1. 此研究主題在於探討公果蠅在求偶被拒之後，在酒精攝食量的改變。
2. 對於學生在實驗研究上的辛勞給予嘉許。
3. 本研究發現 NPF 有可能是控制果蠅情緒的神經傳導物質，深具實驗創新性。