THE OVIPOSITION BEHAVIOUR OF THE BLACK VINE WEEVIL *Otiorhynchus sulcatus* (FABR)

by

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Copyright statement

For My Uncle Ken and Auntie Joan

Abstract

Within the constraints of the experiments conducted vine weevil were found to exhibit a strategy of volume of indiscriminate oviposition rather than a strategy of definitive site selection characteristic of a true oviposition behaviour.

A series of experiments was conducted to determine oviposition and host plant interactions under conditions of choice and absence of choice of host plant. It was found that oviposition was greatest for Yew, followed by polyanthus, fushcia, strawberry and (despite the name) was found to be least on vine plants. Variation was attributed to a combination mainly of ground cover and partially quality of host. Oviposition was found to be much lower under conditions of absence of choice. Variation was attributed to the observation that not all vine weevil oviposit all the time and that feeding and oviposition upon a plant are independent.

A further experiment conducted using strawberry plants with variable leaf N reinforced the interpretation that feeding and oviposition are independent functions. Under conditions of choice of host plant it was found that feeding occurred upon plants that were not capable of generating ova.

Passive transponders attached to individual vine weevil adults were utilised in order to relate oviposition and feeding to specific actions of individual vine weevil. If was found that a greater volume of leaf matter was consumed from leaves of high N that was not associated with an increase in plant visitation. It was also found that the presence of surface refuge increases the volume of oviposition and feeding – possibly as a function of increasing the number of vine weevil that remain in association with the host plant. Direct measurement of leaf area consumed reinforced the observation that feeding and oviposition are not correlated. Transponder signals that indicate that more than one weevil is present at

an oviposition site suggests that vine weevil do not have the need to be alone when ovipositing.

The distribution of oviposition is highly influenced by locomotion. Traditional arena based studies suggested the high level of incidence of directional bias. In order to determine the angle of rotation and the path length an item of apparatus was designed and created. The AVOID apparatus enabled the measurement of path and angle without the detrimental effect of an arena wall. The avoid apparatus was used to determine the variables related to locomotion for a comparison of pre-oviposition weevil locomotion with post oviposition locomotion, the effect of nutritional state upon locomotion and the effect of crowding. Observation suggests that vine weevils that have oviposited have a greater tendency to remain inactive than pre-oviposition vine weevils, which may result in an increased level of dispersal of pre-oviposition vine weevils.

The contribution to dispersal by larval stage *O. sulcatus* was determined. It was found that third instar vine weevil larvae are highly mobile. It is theorised that the artificial barrier created by a plant pot prevent dispersal and, thus, exacerbate the larval status as a pest.

An attempt was made to develop a molecular biology technique to differentiate the ova produced by multiple vine weevils at an oviposition site. It was determined that sufficient DNA could be extracted from a single ovum to enable several PCR reactions. A theoretical model of the inheritance of mtDNA molecules was created to test the feasibility of mtDNA mutation as a differentiation method.

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University Award

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Signed: 

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Chapter 1. - The Biology and Life Cycle of *Otiorhynchus sulcatus*

Otiorhynchus sulcatus (FABR) is a member of the order Coleoptera (beetles) and follows the typical adult form of a heavily sclerotized and depressed body, with the forewings modified as rigid elytra. In *O. sulcatus* the elytra have fused and the hind wings have been lost rendering *O. sulcatus* flightless.

O. sulcatus is also a member of the family, Curculionidae, a family of small to medium sized coleopterans characterised by a distinct rostrum on the head. Other members of this family include the economically important bark weevil (*Aesiotes notabilis* Pascoe), cotton-boll weevil (*Anthonomus grandis grandis* Boheman), grain weevil (*Sitophilus granaries* Linnaeus) and rice weevil (*Sitophilus oryzae* Linnaeus). The adult *O. sulcatus* has a typical curculionidian form and is approximately 9-12mm in length. *O. sulcatus* is dull black in colour with small tufts of yellow "hair" on the abdomen and elytra which give a yellow speckled appearance (Willis, 1964).

O. sulcatus has a non-reproductive period directly after adult emergence. This stage, often referred to as the pre-oviposition period, may be a true adolescent process of ovary maturation. Once ovary maturation has occurred the vine weevil can produce fertile eggs without the need for mating. *O. sulcatus* is assumed to be an obligate parthenogenetic organism as the male of the species has never been documented (Smith, 1932; Moorehouse *et al*, 1992b). *O. sulcatus* follows the typical holometabolous life cycle of distinct and morphologically different life stages.

The newly laid ova are a sub-spherical ovoid, approximately 0.8-1mm in diameter, and a pale cream in colour. The chorion of the ova then become melanised resulting in a colour

change to an orange-brown colour. The melanisation of the chorion of the ova can be used as an indicator of the viability of vine weevil ova. Non-viable ova do not undergo melanisation (Willis, 1964). It is not known if ova become melanised because they are viable or whether ova become viable because they have melanised.

Larvae are a translucent pale cream in colour with a distinctive tan head with numerous dorsal setae. The position and number of setae can be used to differentiate *O. sulcatus* from similar larvae such as *O. porcatus* (Herbst), *O. singularis* (Linnaeus), *O. ovatus* (Linnaeus) and *O. clavipes* (Bonsdorff) (Willis 1964). The body colour of the larvae may vary due to colour of the food source and also due to infection by entomopathogenic organisms such as *Metarhizium anisopliae* (Metschnikoff) (Moorehouse, 1990). The number of larval instars is currently uncertain. LaLone and Clarke (1981) distinguished six larval instars, however, Smith (1932) identified two larval groups with either six or seven larval instars.

Pupation occurs in a cell constructed within the soil. Pupation, by definition, is a non-feeding period of development where considerable physical changes occur to the organism - a period with a significant increase in biochemical and cellular activity.

1.1. The Historical and Geographical importance of the vine weevil

Otiorhynchus sulcatus is thought by many to be the most serious pest of hardy ornamental nursery stock and soft fruit of the 1980's and 1990's (Alford 1996; Backhaus 1996; Bathon 1996; Blackshaw 1983; Buxton 1996; Frers 1996; Gange 1996; Labuschagne 1994; Moorhouse 1990; Moorhouse *et al.* 1992a&b; Morgan 1996; Mosson *et al.* 1996; Neubauer 1996; Pickett *et al.* 1996; Van Tol 1996a&b; Vogt 1996; Zimmermann 1996). Damage to greenhouse plants by *O. sulcatus* was reported in 1834 in Germany (Bouche 1834). Westwood (1837) subsequently reported vine weevil damage in the UK. Warner

and Negley (1976) attribute a sighting of *Curculio apiculatus* (Gyllenhal) in Massachusetts in 1831 as *O. sulcatus*. Despite the earlier sighting in America the origin of the genus Otiorhynchus is believed to be European where the fossil record of the genus dates back to the pleistocene (Feytaud 1918). From Europe the vine weevil is believed to have spread to other parts of the globe through the shipment of infected plants (Smith 1932). Historically the effect of *O. sulcatus* has been confused by multiple scientific and common names. The genus *Otiorhynchus* is often misspelled as *0tiorrhynchus. O. sulcatus* was known as *Brachyrhynchus sulcatus* in the USA from 1920 until 1972.

O. sulcatus is endemic to the temperate areas of Europe from northern Italy to southern parts of the Baltic states and is believed to extend into central Russia (Anon 1974). In North America, *O. sulcatus* is mainly found in the eastern and western states with small "islands" of infection further into the continent (Warner and Negley 1976). Similarly *O. sulcatus* is found in the eastern and western provinces of Canada (Warner and Negley 1976). One of the earliest observations of *O. sulcatus* in the southern hemisphere was made by Kingsley (1898) in New Zealand. Since that time *O. sulcatus* has also been reported in south eastern Australia and Tasmania (Anon, 1974). Masaki *et al.* (1984) reported the recent arrival of *O. sulcatus* in Japan. *O. sulcatus* has also been reported in Chile by Prado (1988). Unusually *O. sulcatus* has not been reported in South Africa.

The recent rapid increase in the pest status of *O. sulcatus* is linked to a number of modern husbandry practices. The weevil populations on strawberry plots that were covered with a black polythene mulch were twenty times greater than on similar plots without the mulch (Stenseth 1979). The widespread use of peat and bark based potting mixes, and the expansion of horticultural production, is also likely to have influenced the frequency of weevil infestation (Blackshaw and Thompson 1993; Nielsen and Roth 1985; Stimmann *et*

al. 1985). It has also been suggested that the extensive use of insecticides has disrupted the balance between *O. sulcatus* and its natural enemies (Evenhuis 1978).

1.2. Chemical control of vine weevil and resurgence as a pest

The banning of aldrin in the UK in 1989 (later than in most European countries) resulted in the re-establishment of *O. sulcatus* in many areas at near epidemic proportions (May and Ellis 1996). Until 1989 it was common practice in the UK to incorporate aldrin into growing media for most container grown hardy ornamental nursery stock and other horticultural plants (May and Ellis 1996). This method of application requires minimal resources, little timing or planning and requires very little specialist equipment for use. In a study of nine pesticides applied in this way to the growing media of polyanthus plants only aldrin gave 100% control (Blackshaw 1983). In the same study it was shown that complete control could only be achieved by the other insecticides using a periodic soil drench. A soil drench system of insect control would require greater planning and investment in capital equipment for application. In a later study (Blackshaw 1984) aldrin was further vindicated as the only currently available effective compost treatment. Even when applied as a drench the other insecticides were not as persistent as aldrin and did not sustain effectiveness throughout the experiment (Blackshaw 1984). The development of a controlled release formula of chlorpyrifos that could be used effectively (at high dose rates) in the "traditional" control methodology has been heralded as an effective alternative to aldrin (May and Ellis, 1996). Initial trials however found that this technology was not effective against vine weevil larvae, possibly due to the particle size being too large and insufficient active ingredient release (Blackshaw 1986; Blackshaw 1987; Blackshaw and O'Neill 1987). The suggestion of smaller particles has since been adopted and the technology marketed to growers as suSCon Green© (May and Ellis 1996). Products containing imidacloprid now dominate the market (R P Blackshaw, 2003 *personal communication*).

1.3. Host range and damage caused by the vine weevil

O. sulcatus is highly polyphagus. Smith (1932) compiled a list of 77 host plants that the adult stage would feed upon. The USDA Plant Pest Survey Files include a further 70 species (Warner and Negley 1976). In tests of important Japanese species, the adults fed on 101 out of 108 species with 46 out of 49 families represented (Masaki *et al.* 1984). As with many insect species the food type and host range vary with life stage. Adult vine weevils feed on leaf material producing a characteristic notch in the leaf margin. Larvae feed on the root system of the plant. It should be noted however that a demonstration of adult feeding on a host plant does not mean that *O. sulcatus* can complete its life cycle on that species (Moorhouse *et al.* 1992b).

Infestation by *O. sulcatus* can result in substantial levels of crop damage. A loss of £10,000 by a Comish nursery was reported in the Grower (Anon 1996a). Mason (1960) reported the loss of over 1000 cyclamen plants at one nursery in the autumn of 1952. Serious damage has also been reported on a wide range of plant species; including *Taxus*, Rhododendron, *Ribes* and *Frageria*. (Moorhouse *et al.* 1992b).

The damage reported on most species results from the feeding activity of larvae on the root system (Penman and Scott 1976). An exception to this is damage to grape vines where loss of berries and reduced berry weights followed damage to the berry particles and cluster stems by adult weevils (Cone 1963). In later work Cone (1968) observed complete defoliation by 11 adults per vine and calculated an economic threshold of 1-3 adults per plant. During this study, injury resulting from larval feeding over a three-year period, did not significantly reduce yield.

The damage threshold, however, appears to be host species and host age specific. LaLone and Clarke (1981) found that the damage threshold (defined by the death of the host plant) for young Rhododendron can be as low as three larvae. Established plants are more resistant to weevil damage than young plants and newly transplanted cuttings because their larger root systems can withstand some feeding without adverse effects on growth (Neiswander 1953). Penman and Scott (1976) determined a positive correlation between larval numbers and damage to strawberry plants. Severely damaged plants had fewer leaves, a smaller leaf area and less berries. They estimated that the economic threshold was between two and eight larvae per plant. They also noted the effect of adult feeding and concluded that this only had a significant effect upon plants already severely damaged by high larval populations. The position of the larvae within the root system will also have a significant effect on damage level (Evenhuis 1978). One larva can do more damage at the base of the stem than several around the periphery of the root system.

Vine weevils can also indirectly affect the economics of hardy ornamental nursery stock and soft fruit production. *O. sulcatus* can transmit the plant virus associated with panachure of grapevines (Ochs 1960). *O. sulcatus* can cause aesthetic damage to plants by adult feeding.

Contamination of nursery stock can also have qualitative repercussions, resulting in rejection, with subsequent reduction in repeat/return business. Berry contamination, if the crop is harvested mechanically, can also have serious economic repercussions to soft fruit growers (Shanks 1981).

1.4. The Effect of Temperature Upon Vine Weevil Development and Life Cycle

1.4.1. Hatching and ova development

The relationship between incubation and temperature is not linear (Montgomery and Nielsen 1979). Montgomery and Nielson describe the relationship between incubation period and temperature using the equation:

$$Y = 93.26 - 6.54T + O.13T^2$$

Where: Y = Development time in Days, and T = Temperature in °C

Development time decreases with increase in temperature up to 25°C, however, as the temperature exceeds 25°C incubation time increases slightly. The survival of eggs is greatly reduced at temperatures greater than 27°C (Montgomery and Nielsen 1979). The temperature dependency of hatching was also demonstrated by Stimmann *et al* (1985) where they observed a hatching time of 10 days at 75°F (23.89°C) (Stimmann *et al*, 1985). (The hatching time predicted by the Montgomery equation is 11.21 days at 23.89°C.)

At 25°C most eggs melanise (Montgomery and Nielsen 1979). However if held at 31°C for the first 12 hours after being laid melanisation (and survival) is greatly reduced. If the 12 hour exposure is delayed until eggs are 24 hours old then the severity of the reduction of survival is not as great. Prolonged exposure to 31°C will kill all eggs. Montgomery and Nielsen (1979) also noted that a regime of 8 hours at 30°C followed by 16 hours at 20°C for 14 days resulted in the non-viability of all the eggs treated (Montgomery and Nielsen 1979).

The relative susceptibility of newly laid eggs to temperature extremes has also been noted by Shanks and Finnigan (1973) who demonstrated that newly laid eggs are more susceptible to extremes of temperatures than eggs three to four days old. Constant temperatures above 26.7°C and below 15.6°C greatly reduced hatching. One or more exposures of three or six hours at 26.7°C or 32.2°C interspersed with 15.6°C had less effect (Shanks and Finnigan, 1973). Despite the narrow range of ideal temperatures observed a minimum feasible development temperature for eggs of 6°C has been demonstrated (Stenseth 1979).

1.4.2. Pupation and larval development

Pre-adult development took 200 days at 15°C and 130 days at 24°C (Stenseth 1979). Eggs and larvae develop at 27°C but pupation does not take place. The minimum development temperature is 2°C to 6°C for larvae and pre-pupae and 12°C for pupae (Stenseth 1979). Pupation has been identified as the most temperature sensitive stage of vine weevil development.

1.4.3. Oviposition and adult responses

Vine weevils are more fecund at a constant temperature of 20°C than under an outdoor regime of temperature variation (Cram, 1964). At 20°C the oviposition period was completed after 30 weeks of constant oviposition. However outdoors oviposition stopped for overwintering. This was defined as overwintering rather than as true diapause by the fact that the vine weevils resumed ovipositing when introduced to a constant 20°C temperature regime (Cram, 1964). Evenhuis (1978), however, noted a variable effect due to reintroduction to higher temperatures. At various temperatures some of the weevils

resumed ovipositing after a few days, some took up to four weeks and some never resumed oviposition. Evenhuis (1978) stated that the range of oviposition temperature tolerance was determined as 11°C to 26°C with the largest number of eggs laid between 20°C and 23°C. Oviposition in natural populations, however, commences at temperatures below the 12°C minimum postulated by Stenseth (1979) as determined by the initialisation date of oviposition (Blackshaw, 1992). Weevils kept at 8°C and then placed at -5°C for up to 4 hours suffered no harmful effect (Evenhuis, 1978).

The length of the pre-oviposition period is also temperature dependant. Stimmann *et al* (1985) state that adults require 600 day-degrees[1] Fahrenheit (316 day. °C) before they begin to produce ova.

At a population level the total number of eggs laid in a growing season decreases in relation to the number of days with mean subzero temperatures since the previous egg laying season (Blackshaw, 1996). This effect was postulated as winter severity acting upon overwintering adults. Initiation date of oviposition, and pre-oviposition period, could also be related to the number of days with mean subzero temperatures (Blackshaw, 1996).

1.5. The Effect of Humidity Upon Vine Weevil Development and Life Cycle

Low humidity has a significant effect upon ova and early larval stage survival. A constant relative humidity (RH) of less than 65% decreases egg viability to zero. First stage larval survival, however, is greatly reduced with RH of less than 85% (Shanks and Finnigan, 1973). Shanks and Finnigan (1973) found, however, that a six hour exposure to 33% RH

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[1] The cumulative mean daily temperature the adult would need to experience before oviposition would commence.

has no effect upon the viability. Even five exposures to this protocol had little effect upon the viability of ova (Shanks and Finnigan, 1973).

The direct physical effect of low humidity upon ova and larvae is attributed by Montgomery and Nielsen (1979) to the drying effect of the air resulting in dehydration. The drying effect of the air due to relative humidity can be expressed by the concept of pressure saturation deficiency. This is defined by the difference in vapour pressure between the actual relative humidity and the expected vapour pressure at 100% RH at a given temperature. Montgomery and Nielsen (1979) noted that as deficit increases the embryonic development increased at an accelerating rate. This effect is not linear and was defined by the equation:

$$Y = 13.57 + 0.047D + 0.069D^2$$

Where: Y = Development time in days, and D = Saturation deficit.

Montgomery and Nielsen (1979) also noted that if the deficit exceeds 4.5 mm(Hg) then ova survival is greatly reduced with a maximum of 6.5mm(Hg) deficit resulting in 100% mortality of ova.

The combined effect of temperature (T) and vapour pressure deficit (D) was described by Montgomery and Nielsen (1979) using the equation:

$$Y = 73.55 - 4.74T + 0.438D + O.0891T^2 + O.0963D^2 - O.0337TD$$

Low humidity at an oviposition site can also have an indirect effect upon larval survival. Shanks and Finnigan (1973) found that first stage larvae cannot penetrate an air-dried soil layer greater than 15mm (Shanks and Finnigan, 1973).

Not only do ovipositing adults actively seek out humid resting sites, but they also retain their eggs under dry conditions, until conditions improve. The availability and quality of resting/oviposition sites may be more critical than air conditions to oviposition and reproductive success (Montgomery and Nielsen, 1979).

1.6. The Effect of Host Plant Upon Vine Weevil Development and Life Cycle

Cram and Pearson (1965) stated that the number and viability of the eggs produced by an adult vine weevil is dependant upon the host plant. This view is supported by Penman and Scott (1976) who found that the mean egg production on clover of 155.4 eggs was significantly less than the 506.0 and 543.6 mean egg production of vine weevil adults fed strawberry and blackberry respectively. Shanks (1980) found that adults fed a *Frageria x Ananassa duchesne* variety of strawberry; produced more eggs, with a higher viability, and produced progeny that were more likely to develop into adults, than *Taxus media* (Rehder) fed adults.

Varietal differences have also been shown to significantly affect development. Cram and Daubeny (1982) noted that, in experiments with eight raspberry cultivars, significant differences in; pre-oviposition period, number of eggs laid and relative number of fertile eggs occur. Cram (1980) noted similar effects upon the number of eggs produced and viability of eggs due to cultivar in strawberry. This was reinforced by Shanks and Doss (1986) who demonstrated a difference in reproductive success between the strawberry

cultivars 'totem' and a clone of the wild beach strawberry (*Frageria chiloensis* L.) designated CL-5.

1.6.1. Host plant as nutrient source

If we view the host plant simply as a nutrient source for the creation of ova then it is to be expected that a number of development and reproductive events will be directly affected by host nutritional quality. A more nutritious host should reduce development time and increase reproductive success. Cram and Pearson (1965) observed that the rate of weight gain during the first three weeks after eclosion varies significantly between vine weevils reared on different host plants. The weight gain before oviposition, however, was not found to be significantly different for different host plants (Cram and Pearson, 1965). This would seem to suggest that there is a correlation between the rate of weight gain and the length of the pre-oviposition period. This view was later reinforced by Cram (1980) who found further evidence of a correlation between the rate of weight gain and the length of pre-oviposition period. Cram and Daubeny (1982), however, found that the rate of weight gain in post eclosion adults was not significantly different for eight different raspberry cultivars which did show differences in pre-oviposition period and fecundity.

Maier (1981) observed that plant species that support the shortest pre-oviposition period also support the highest rate of egg production. Penman and Scott (1976), however, did not observe a difference in pre-oviposition period between three hosts even though the hosts produced significantly different reproductive success rates.

Doss and Shanks(1985) found that adult vine weevils change feeding pattern with age. Heaviest feeding occurs ten weeks after eclosion (Doss and Shanks, 1985). They also found that selectivity of food source, between "alpine" strawberry and nutrient

impregnated discs is minimal in the first three weeks after eclosion. Doss and Shanks (1985) also demonstrated that one year post-eclosion adults ate less of a sucrose impregnated disk than three week post-eclosion adults.

The same authors noted that the increase in pre-oviposition period and the lower reproductive success of adults fed CL-5 variety of strawberry could be partially attributed to the fact that daily intake of the variety CL-5 was lower than that of the other variety tested, 'Totem' (Shanks and Doss, 1986).

Adults fed whole strawberry leaves have shorter pre-oviposition periods than adults fed a 2.1cm diameter disk cut from a leaf of the same plant. Even when the area of the leaf and disk eaten were the same this effect still occurred. This process was attributed to the fact that, when presented with whole leaves, the weevils tend to be more selective and feed at the leaf margin, where the leaf veins are smaller (Shanks and Doss, 1986). If there were a difference in the nutritional quality between the leaf margin and the leaf disks then this would support the mechanistic concept of direct conversion of ingested nutrients into ova. If, however, the reduced oviposition is a reaction to the damage to the leaf then this could form the basis of a discriminatory process whereby vine weevil discriminate against damaged host plants. Cram (1980), however, found that there was no correlation between the leaf area consumed by an adult and the number of eggs produced. The clones CL-5 and GCL-8 reduced feeding and increased pre-oviposition period in newly eclosed adults (Shanks *et al*, 1984). Shanks *et al* (1984) also found that egg production was closely correlated with the amount of feeding on a particular clone.

The relationship between oviposition and nutritional quality of host plant is most evident in nitrogen supplementation studies. Cram (1965a) demonstrated that the length of the pre-oviposition period decreased from 53 days to 33 days if the rate of application of N to host

plants increases from 26 ppm to 210 ppm. Cram (1965a) also found that the number of eggs produced and the viability of eggs increases from 146 eggs with a 44% viability, at 26 ppm N, to 506 eggs with a 74% viability at 210 ppm application of N. Cram (1965b) later discovered that the form in which the N is applied does not significantly affect the fecundity and viability.

The fecundity of weevils increases with increasing N content (Maier, 1981). The effect of N content influencing fecundity is not as apparent in interspecific differences as they are in intraspecific differences in host plant (Maier, 1981). This was attributed by Maier (1981) to leaf composition, toxins and differences in toughness of the leaf of different host plants.

Hesjedal (1984) found that the N content of host leaf has a significant effect upon fecundity. This effect was apparent even with a reduction from 2.2 - 2.0% N dry leaf matter. Hesjedal (1984) also found a positive correlation between "digestion" of N and fecundity (where digestion is calculated from the amount of "wet" leaf consumed multiplied by the mg dry matter content of N of an equivalent leaf). This concept may explain why Cram (1980) did not find a correlation between leaf area consumed and egg production. Hesjedal (1984) also found that the amount of N intake required to produce one egg is the same for all host's tested.

There was not a behavioural response by the adults to increase the volume of ingestion of leaves of low N levels to compensate for the lower nutritional value - egg production simply decreased with decreasing nitrogen levels (Hesjedal 1984).

Shanks and Doss (1986) stated that antixenosis cannot fully account for differences in pre-oviposition period and reproductive success. Even with equivalent daily food intake, pre-oviposition period is longer and the reproductive success is reduced when fed with the CL-

5 clone (Shanks and Doss, 1986). Non-nutrient content related mechanisms must therefore be acting. Reduced feeding by adult vine weevil was attributed by Doss *et al* (1987) to surface differences in the CL-5 clones, as a membrane filter assay demonstrated that there is not a chemical basis for feeding resistance. Adult feeding on the clone CL-5 is negatively correlated with "hair" density (Doss and Shanks, 1988). However in later work with CL-5 Duchesne x only 1% of vine weevil resistance could be attributed to leaf pubescence (Doss *et al*, 1991).

Direct toxic or anti-nutritional factors may cause deviation from a direct relationship between quality of host as a nutrient source and oviposition. Some plants, for example Brassica spp, produce aromatic isothiocyanates. Isothiocyanates are highly toxic to vine weevil ova (Borek *et al*, 1995). Adult survival on clover (which contains high levels of protein) was reported to be less than on strawberry and blackcurrant; however, the data to substantiate this observation was absent from this publication (Penman and Scott, 1976).

Larval survival can be affected by the host plant. A survival rate of 17% was recorded on larvae reared in azalea (Hanula, 1988). The weights of adults at emergence were also 40% lower on azalea reared larvae than those reared on strawberry or *Taxus*. Diflubenzuron at 0.013-0.05g ai.litre can reduce fecundity by 15-81% without affecting adult longevity (Zepp *et al*, 1979). A strategy based only upon a mechanistic concept of seeking high N (or other nutrient) based host plants would, therefore, be disrupted by these factors.

Vine weevils are complex organisms capable of complex behaviour patterns. Thus to understand the effect of the mechanistic concept one must not only consider the absolute nutritional value of the food source but also the perception of the value of the food source by vine weevil.

In laboratory experiments Doss and Shanks (1985) demonstrated that the area of an artificial membrane eaten by adults is linearly related to sucrose concentration. The presence of sitosterol stimulated a proportional response in area of artificial membrane filter eaten by adult vine weevil (Doss and Shanks, 1984). Sitosterol was believed to be acting as a lipid phagostimulant. Sucrose, when used with sitosterol, had a synergistic effect in promoting vine weevil feeding (Doss and Shanks, 1984).

Vine weevil can be deterred from feeding upon certain hosts using a cinnamic acid derivative. A 1% w/w solution applied to strawberry leaves reduces feeding by 65% when adult vine weevils are given a choice of host (Mosson *et al*, 1996).

Vine weevil adults exhibit a choice in oviposition host. Hanula (1988) found that ovipositing adult vine weevils had a high preference for *Taxus* when given a choice of host plants. Previous host experience has an effect on oviposition preference. Hanula (1988) found that 13% more eggs were laid on *Euonymus* if *Euonymus* was the pre-oviposition host. Also, if larvae are reared on azalea roots then they laid 21% more eggs on azalea foliage than weevils from non-azalea in choice tests (Hanula, 1988).

Hanula (1988) reports that, even when sensory structures of adult weevils are destroyed, more eggs were laid on *Taxus* than on other host plants when a choice of host plant is given.

1.7. Cyclical oviposition

A number of authors have noted a cyclical pattern to oviposition. Penman and Scott (1976) differentiate a cyclical egg production process which is dependant upon host plant. The mean number of egg laying days per cycle varied from; 12.2, 5.11 and 49.2 when fed

the host plants clover, strawberry and blackberry respectively. The period of time between cycles is also affected by host plant choice: Clover (12.8 days), strawberry (8.7 days) and blackberry (15.0 days). This view is refuted by Nielsen and Dunlap (1981) who deny that Penman and Scott's data shows cyclical egg production.

The number of eggs produced per cycle appears to be entirely dependant upon the cycle length as there is not a significant difference in the number of eggs produced per day of the cycle: Clover (56.1 eggs per cycle, 4.6 eggs per day), strawberry (285.9 eggs per cycle, 5.59 eggs per day) and blackcurrant (214.6 eggs per cycle, 4.36 eggs per day) (Penman and Scott, 1976). This cyclical process could be explained by a period of ovary maturation, which is host plant dependant, or more directly from an effective reduction in intake of bioavailable plant material.

The proportion of vine weevil with mature ovaries peaks in early June and then again in early to mid August. The peak number of adults occurs in early July. This twin peak was attributed to an overwintering and a newly formed adult population occurring where the adult population consists mainly of newly emerged adults (Hanula, 1990). Hanula (1990) observed a delay of a month between peak adult vine weevil capture and a peak in the number of weevils captured with mature ovaries.

Blackshaw (1996) also demonstrates a trough in oviposition - number of eggs found - which occurred in either August or July. This trough was attributed to the overwintering adults contribution to oviposition.

1.8. *The effect of substrate upon vine weevil development and life cycle.*

The substrate can have a number of effects upon development and survival of vine weevil. Nielsen and Roth (1985) noted that container media can have a highly variable and significant effect upon vine weevil larvae mortality. Nielsen and Roth (1985) attributed this to the effect of media upon moisture content. Garth and Shanks (1978) found that the greatest infestation rate (i.e. highest survival rate) occurs when eggs were buried 2.5cm deep. Thus the probability of survival is increased when oviposition occurs within the substrate rather than upon the surface (Garth and Shanks, 1978). Hanula (1993) observed that 90% of the larvae feeding on *Taxus cuspidata* were found in the top 15cm of substrate with a maximum depth of 30cm. The distributions of pupae follow a similar pattern. Frers (1996) observed that vine weevil adults, when ovipositing, express a relative preference for certain surface media. In a choice test of surface media vine weevil laid 151 eggs on peat moss, 74 eggs on sand and 13 eggs on bark mulch. Blackshaw and Thompson (1993) found that the numbers of larvae increased with higher ratios of bark to peat in composts but declined as the size fraction of the bark used increased. It was suggested that the bark may promote more vigorous growth in the host plant, polyanthus, and thus enable plants to support more larvae (Blackshaw and Thompson, 1993).

1.9. *Photoperiod and vine weevil oviposition*

Weevils can be induced to return to ovipositing in the laboratory using a 16h photoperiod (Nielsen and Dunlap, 1981). Garth and Shanks (1978) found that vine weevil ceased ovipositing when the photoperiod decreased from 16h to 12h. Oviposition resumed, however, after four weeks. Garth and Shanks (1978) concluded that photoperiod length is not the only controlling factor in oviposition cessation and resumption. Blackshaw (1992),

however, found no evidence that temperature or day length limited oviposition in the autumn. The relationship between the number of eggs produced per week and temperature and day length may reflect the timing of recruitment in the adult population, rather than climatic dependency (Blackshaw, 1992).

1.10. Other Factors that Effect Vine Weevil Development and Life Cycle

Blackshaw (1996) reported a correlation between the viability of eggs and the number of eggs produced. These are related by the equation:

$$V=9.2+8.84N$$

Where: V = arcsine transformed percentage viability, and N = Square root of the number of eggs laid

A correlation between larval density and mortality has also been observed by La Lone and Clarke (1981).

1.11. Olfaction and behaviour

Olfaction may have an influence upon the oviposition behaviour of the vine weevil. Pickett *et al* (1996) demonstrated the existence of a substance that stimulates the aggregation of vine weevil adults. Van Tol *et al* (2002) demonstrated a positive choice for damaged *Euonymus* over clean air – suggesting that green leaf volatiles may act as attractants to vine weevil. This observation was reinforced by the observation of electroantennogram sensitivity in vine weevil to green leaf volatiles (Van Tol and Visser, 2002). The effect of volatiles upon oviposition site choice has not been determined.

1.12. Aims and objectives

The purpose of the study was to investigate vine weevil behaviour that may affect oviposition site choice or influence the spatial distribution of ova. The study can be broken down into five principle areas:

- Vine weevils are notoriously polyphagus. Under conditions of choice of host plant, do vine weevils express a preference for ovipositing upon a specific host species? Is choice of oviposition site influenced by host nutritional quality? One aim was to investigate the effect of host choice upon relative oviposition.

- Does the quantity of ova found at a host site related to the amount of time spent in association with that host plant? Are vine weevils territorial? An objective of this study was to investigate and develop techniques for tracking vine weevils. Once this was achieved it was the aim to determine the relationship (if any) between residency time and oviposition.

- Does the vine weevil exhibit a particular locomotory pattern that optimises search and (oviposition) site utilisation? An objective of the study was to investigate and develop techniques for observing vine weevil locomotion with the aim to measuring locomotory response (if any) to physiological and environmental factors.

- Are the ova found at a particular site the product of single or multiple vine weevil adults? One objective was to investigate the feasibility of isotopic labelling and molecular genetic methods of differentiating the ova produced by (potentially clonally related) vine weevils with the aim of differentiating the parental source of

ova at an oviposition site.

- Is there an actual need for detailed and explicit oviposition behaviour in vine weevil to ensure the optimal distribution of larvae? How important is larval locomotion to larval distribution? The objective was to investigate and develop techniques for tracking vine weevil larvae with the aim to measuring the rate of dispersal of larvae.

Chapter 2. - The investigation of *Otiorhynchus sulcatus* oviposition host and substrate choice within a large arena

2.1. Introduction.

Fecundity, viability of ova and development are highly dependent upon host type (Cram and Pearson, 1965, Penman and Scott, 1976, Shanks, 1980, Cram and Daubeny, 1982, Cram, 1980, Shanks and Doss, 1986, Maier, 1981, Penman and Scott, 1976, Hesjedal, 1984). Given this variation in host quality – does *Otiorhynchus sulcatus* express a choice for the higher quality host for an oviposition site?

Survival of larvae is also dependent upon substrate (Nielsen and Roth, 1985, Garth and Shanks, 1978, Blackshaw and Thomson, 1993). Frers (1996) observed oviposition substrate choice by vine weevil in choice tests. Given the variation in substrate quality and the apparent choice of substrate exhibited by *O sulcatus* – how is oviposition affected by a combination of host and substrate choice?

Two experiments were conducted. The first trial investigated the effect of host plant and substrate upon relative oviposition rates, under conditions of choice and non-choice. The second trial involved the manipulation of %N in the leaf, thus creating a controlled variation in host plant quality, and the observation of the effect of specific host quality upon relative oviposition rates. Strawberry was used as the host for the second experiment as it is the principle host used for variable N trials in non-choice experiments in the literature (Cram, 1965a).

2.2. Methodology

2.2.1. *Very Large Arena*

Choice experiments were conducted within an outdoor arena constructed at the Seale Hayne Campus of the University of Plymouth.

The arena consisted of boxed area 2.5m x 3.5m x 1m, with a triangular roof 1.5m above the box at the apex. The roof area and door was covered in a 2mm mesh to prevent the escape of adult vine weevil. The box section was buried to a depth of 30-35cm to prevent escape of any larvae from the arena.

Fifty adult *O sulcatus* were introduced into the arena in April 1997 and a further fifty added in April 1998. The vine weevil used in this experiment were from a collection of vine weevil adults maintained in the lab established in 1997. This collection was originally populated with adult weevils from multiple sources. Two hundred were supplied by Louise Labuschagne from insectary reared and field collections with a further 27 from samples provided by colleagues and keen gardeners in the South West of England. Subsequent to 1997 the collection was supplemented with adults reared from larvae fed either on polyanthus plants or carrot pieces. The specific provenance of each vine weevil adult could not be determined.

2.2.2. *Small arenas for non-choice experiments*

Non-choice experiments were conducted within 40cm diameter by 40cm high arenas (50 litre buckets) kept adjacent to the VLA and with lids made from the same 2mm mesh as the VLA and 2mm drainage holes drilled into the base.

2.2.3. Extraction of ova from substrates

The surface 2.5cm of substrate was removed from each host plant and placed into a labelled 0.5 litre container. Fresh substrate of the same type was then used to replace that which was removed.

The containers were then returned to the laboratory and, in the case of the substrate "500µm sieved sand", ova were extracted using the Blackshaw method. This involves wet sieving the substrate through a 500µm sieve, the remainder floated upon a solution of saturated magnesium sulphate solution and the suspended ova transferred to a 1ml microfuge tube (Blackshaw, 1992, Blackshaw, 1984). All other substrates were also wet sieved through a series of 5mm and 1mm mesh.

2.3. VLA Experiment 1: Comparison of oviposition upon five host types, five substrate types with choice and without choice.

2.3.1. Method

The five host types; strawberry (cv Red Gauntlet), *Taxus baccata*, polyanthus (cv crescendo), vine, hardy fushcia (cv Mrs Popple), were potted in peat based potting compost in 15cm pots with one of the five substrates; composted bark, 500µm sieved sand, Hydroleca® (light-weight surface dressing approximately 10mm in diameter), gravel (nominally >5mm) or cocoa chippings, covering the top 2.5cm of the pot. 25 plants consisting of each of the five hosts with each of the five substrates were arranged in a graeco-latin square design within the VLA. A further 25 plants of identical composition were also placed within the individual arenas with an adult vine weevil per enclosure.

Plants were fed (using Miracle Gro® at manufacturers recommended concentrations) and watered Mondays and Thursdays.

The top 2.5 cm of substrate was removed for analysis every week immediately prior to the Thursday feed. `Collection of substrates to investigate for the presence of ova began in the third week of July 1997 with weekly collections until the second week of September of the same year.

At the end of the trial all host plants were maintained for the following six months when, in February, they were inspected for larvae.

Data were collated and then analysed via Minitab using three one-way ANOVA for variables; week, substrate and host choice. Subsequent to a significant ANOVA *post-hoc* Tukey's tests were used to determine whether pairs of treatment means were significantly different Tukey's test was then used to determine whether the treatment means were significantly different.

2.3.2. Results – oviposition under conditions of choice

Although the analysis did not yield a statistically significant difference in oviposition ($F=8.11$, $df=7$, $P=0.193$), the distribution of oviposition with time followed a pattern of seasonality, with the peak oviposition occurring in mid-August and with a dramatic cessation of oviposition mid-September (see Figure 2.1).

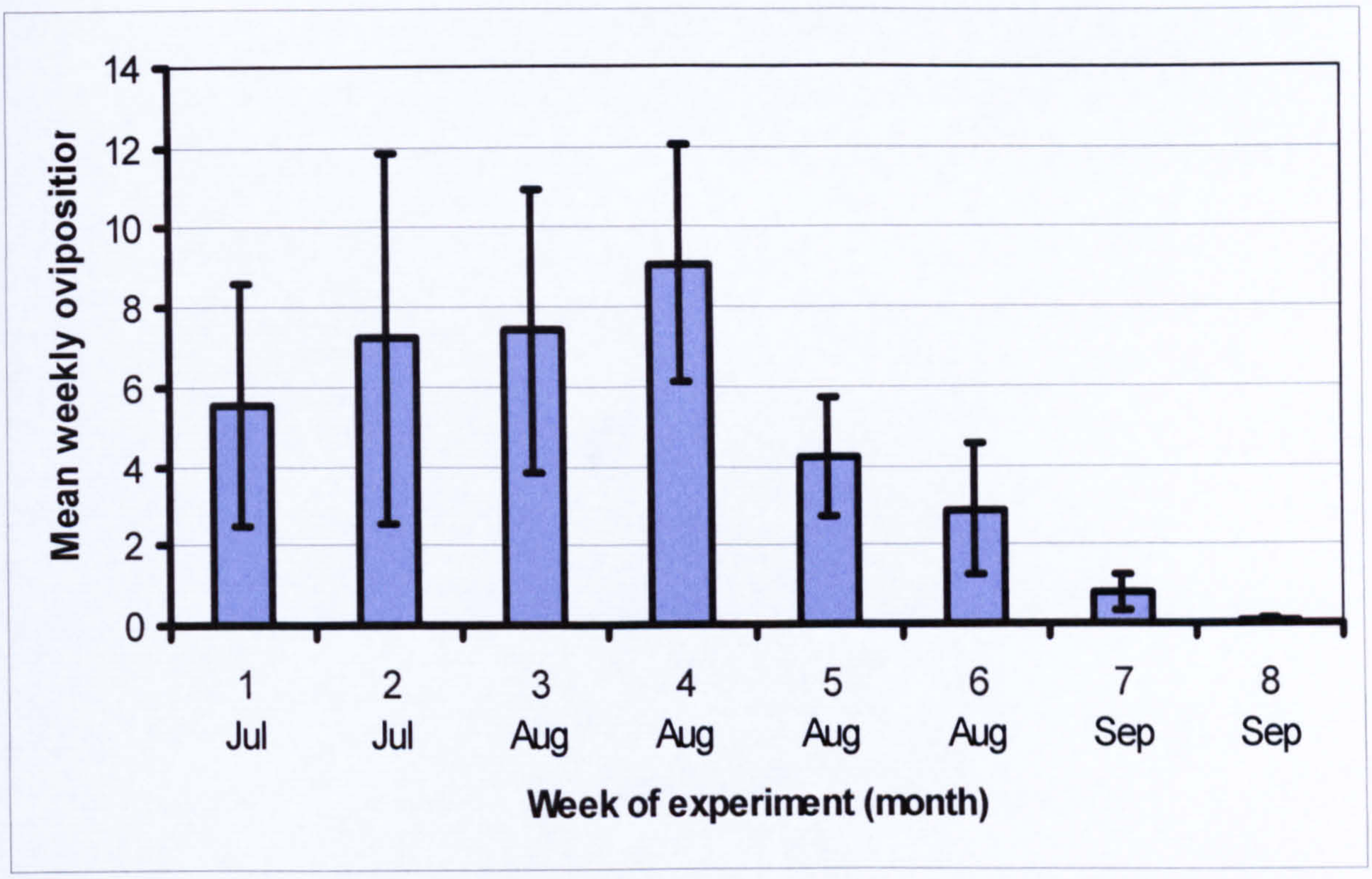

Figure 2.1 – Mean weekly oviposition per host under conditions of choice. Error bars represent one standard deviation. N = 25 host plants

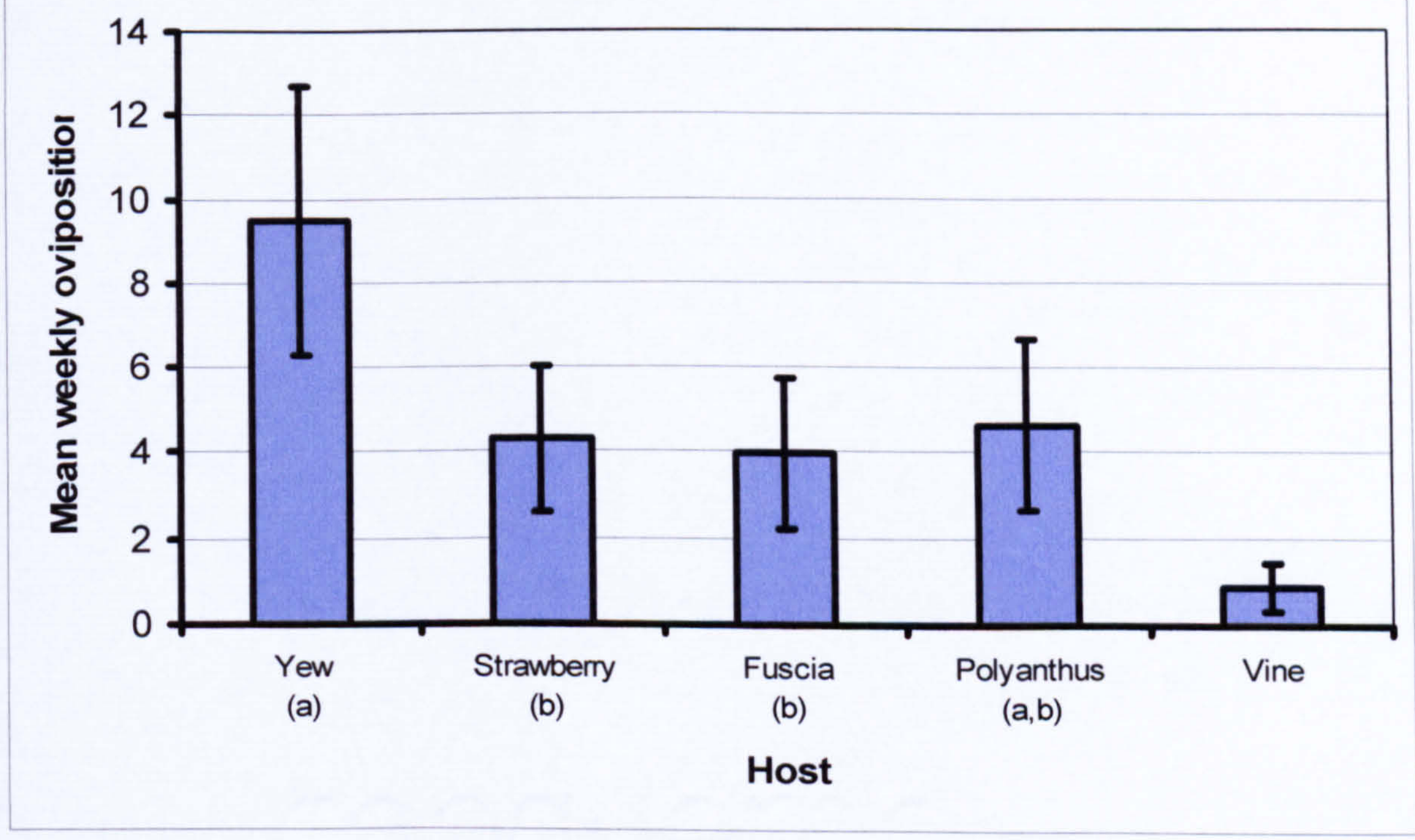

Figure 2.2 – Mean weekly oviposition by host type under conditions of choice. Error bars represent one standard deviation. Figures with the same letter are not significantly different. N = 5 plants of each type.

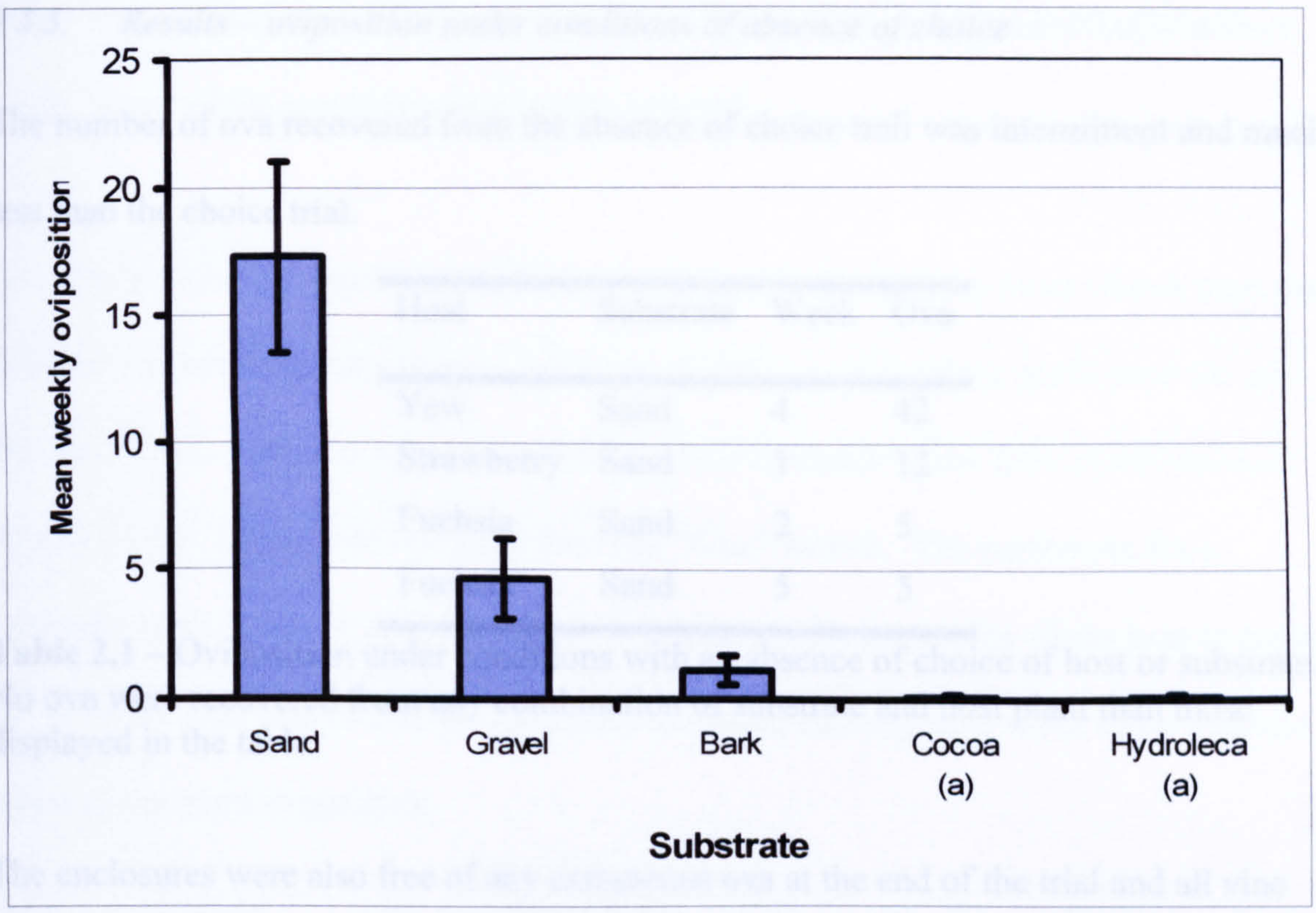

Figure 2.3 - Mean weekly oviposition by substrate type under conditions of choice. Error bars represent one standard deviation. Figures with the same letter are not significantly different. N = 5 for each substrate type.

Host choice also failed to demonstrate a statistical significance over all host types (F=9.21, df=4, P=0.085) see Figure 2.2.

Substrate choice exhibited a statistically significant difference (F=62.63, df=4, P<0.001) between substrates, Tukey test revealed that all substrates were significantly different except between cocoa and Hydroleca®, where recovery was too low to differentiate (see Figure 2.3).

No larvae were found in the choice pots six months after the trial, indicating that all viable eggs had been recovered during the course of the experiment.

2.3.3. Results – oviposition under conditions of absence of choice

The number of ova recovered from the absence of choice trail was intermittent and much less than the choice trial.

Host	Substrate	Week	Ova
Yew	Sand	4	42
Strawberry	Sand	1	12
Fuchsia	Sand	2	5
Fuchsia	Sand	5	5

Table 2.1 – Oviposition under conditions with an absence of choice of host or substrate. No ova were recovered from any combination of substrate and host plant than those displayed in the table.

The enclosures were also free of any extraneous ova at the end of the trial and all vine weevil adults in the non-choice trial remained alive throughout. No larvae were found in the absence of choice pots after the trial.

2.3.4. Discussion

The experiment showed a marked difference between the oviposition under conditions of choice compared to conditions under the absence of choice. The difference is greater than would be expected due to the disparity in population density (choice = two adults per host, non-choice = one adult per host). The disparity cannot be attributed to mortality – the adults under the absence of choice conditions remained alive throughout the trial. The absence of ova found within the individual enclosures suggests that this is a complete absence of oviposition rather than a localised rejection of substrate for oviposition.

The observed disparity in oviposition between choice and non-choice conditions is indicative that not all adults are ovipositing. That, under conditions of choice, the number of ova recovered is the product of a select few individuals, at any given time, rather than all adults ovipositing in concert. Consequently, if the probability of any given adult

ovipositing at any time is sufficiently low, then, the probability of recovering ova from singly caged adults would be significantly reduced.

Host types showed a significant difference between the relative recovery of ova from the different surface substrates under conditions of choice. The strong preference for yew and the relative lack of oviposition upon vine lend credence to the view that *O sulcatus* should be the "*Taxus*" weevil rather than the "Vine" weevil. The preference for a particular host, however, may have little in common with the quality of the host to support larvae or the host plant's nutritional quality to adults, but may have more to do with the shape of the plant in question.

The vines used in the trial were tall and narrow with (proportionately) little foliage at the base of the plant and provide little ground cover. The other plants used in the trial were more bush like – providing ground cover and foliage within more proximal foraging distance from the base of the plant.

Recovery of ova from the different substrates was also highly dependant upon substrate. This may not have been a consequence of choice of the particular substrate for oviposition. Observation during the experiment revealed the great difficulty of extracting ova from any substrate other than the 500μm sieved sand. Relative recovery may, therefore, skew substrate choice results, extend the variance attributable to host choice and critically reduce the probability of recovering ova under non-choice conditions.

Given the variation in recovery of ova from the different substrates in the previous experiment an experiment was conducted to determine the effect of the substrate upon the method of recovery.

2.4. *Investigation into the recovery of ova from different substrates*

2.4.1. Method

A blind trial was conducted where 20, 25 or 30 vine weevil ova were added to 500ml of six substrates; peat based compost, composted bark, 500µm sieved sand, Hydroleca®, gravel (nominally >5mm) and cocoa chippings.

The ova were then recovered using the method previously described.

2.4.2. Results

	Ova recovered			%Recovery				
Expected	20	25	30	20	25	30	Mean	SD
Hydroleca	20	24	33	100%	96%	110%	102%	7%
Sand	20	25	30	100%	100%	100%	100%	0%
Gravel	20	22	25	100%	88%	83%	90%	9%
Compost	18	20	29	90%	80%	97%	89%	9%
Bark	16	17	25	80%	68%	83%	77%	8%
Cocoa	14	12	19	70%	48%	63%	60%	11%

Table 2.2 Ova extracted from a positive blind trial from different substrates using the modified Blackshaw method.

2.4.3. Discussion

Under the experimental conditions observed - only sand provided satisfactory recovery. The results of the other substrates belie some practical difficulties.

The relatively high level of recovery from compost was only feasible at the expense of considerable time. 89% recovery from compost, therefore, would not be feasible under large scale experimental conditions.

Cocoa and bark have a tendency to block the sieves and also bind to the ova when moist. Extraction, at an acceptable level of probability, for an acceptable number of extractions, is not practical.

Although gravel and Hydroleca® appear to be viable; with acceptably high probability of recovery and relative ease of extraction, it was found that settlement may become a problem. It was observed that a number of the ova placed in the pot with the substrate were found in the base of the pot and not suspended within the substrate. Consequently, under field conditions, it is likely that, if any ova are deposited into the substrate, settlement may remove them to the underlying substrate. Additionally Hydroleca® is sufficiently lightweight with sufficiently large interstitial spaces that adult vine weevil may be able to easily pass through and oviposit onto the underlying substrate. If these substrates are used, therefore, a second extraction per pot from a layer of sand placed beneath the substrate aught to be conducted.

The experiment also demonstrated the need to thoroughly clear the sieves between extractions. The higher than possible level of extraction of ova from Hydroleca® demonstrates that some extractions may be supplemented by ova previously retained in the sieve or previously unobserved floating in the solution of saturated magnesium sulphate.

2.5. *Feasibility study into the manipulation of N and protein content of strawberry leaves*

2.5.1. Introduction

Before conducting a trial to measure the effect of N application (and indirectly the protein content of leaves) upon oviposition site choice a feasibility study was conducted. The purpose of the study is to determine the effect of N application upon the % leaf N and the soluble protein of the leaf as determined by the Bradford assay. This data would then be used to determine the correlation between the %leaf N and the Bradford results. The correlation would then be used as a standard for determining leaf N concentrations from the Bradford assay.

2.5.2. Methods

Thirty strawberry plants (cv Red Gauntlet) were potted into a media of 500µm sieved sand in 15cm diameter pots. The plants were then watered daily for two weeks to wash away any nutrients bound to the strawberry roots. Following this treatment the plants were then divided into 10 groups. Each group was then fed a different N-depleted nutrient solution of 10%-100% based upon the Long Ashton formula – see appendix I table I.1 and table I.2.

Each plant was fed 200ml of the requisite nutrient solution once per week then 500ml distilled water added to each plant three days later. After five weeks one healthy trifoliation was removed from each plant for analysis.

Three 5mm diameter discs, from each leaf of each trifoliation, were removed and frozen at minus 70°C. The frozen leaf disks were then ground using a micro pestle and a supernatant created in 60µl of ultra pure water. 45µl of the resulting supernatant was then

transferred to a microplate and incubated at room temperature for five minutes with an equal volume of Bradford reagent (Bradford, 1976). The absorbance at 595nm was then determined and compared with ultra pure water/Bradford reagent blank and two bovine serum albumin (BSA) standards.

The remainder of the trifoliation was then kiln dried, weighed and, together with three BSA standards, the %N determined using a LECO C-N analyser using an EDTA calibration standard.

2.5.3. Results

The response of % leaf N to the application of N to the plant was not linear (see figure 2.4). A number of polynomial equations were fitted to the data. A third order polynomial equation fitted to all data points (not just means) was found to give the best R^2 value. The equation of best fit, with an R^2 value of 0.9795, was:

$$y = 0.0946 + 0.0998513x - 0.0022514x^2 + 0.0000183x^3$$

Where y = % leaf N, and x =%N applied to parent plant.

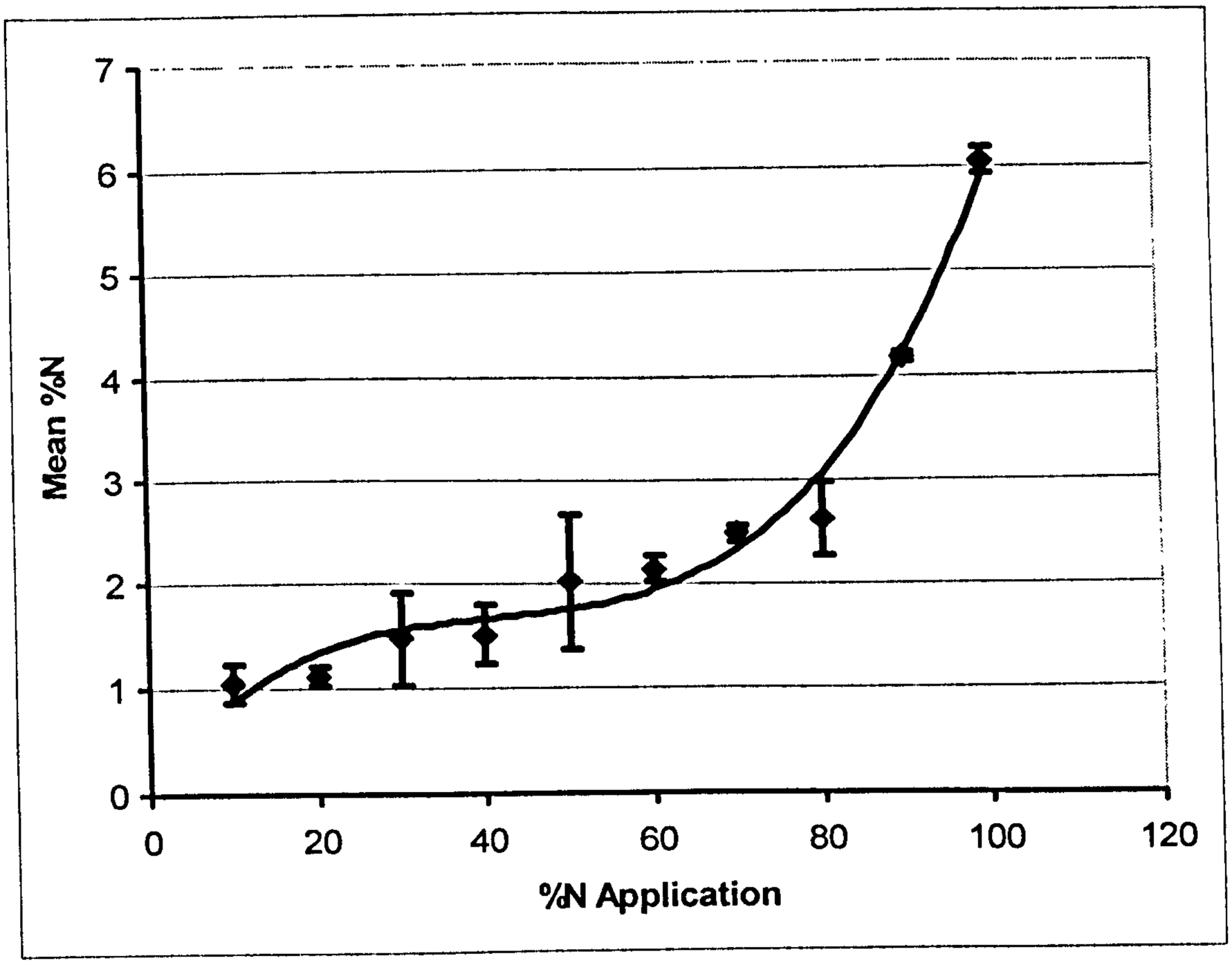

Figure 2.4 - Relationship between leaf %N, as measured using LECO C-N analyser, with varying N application. Error bars represent one standard deviation.

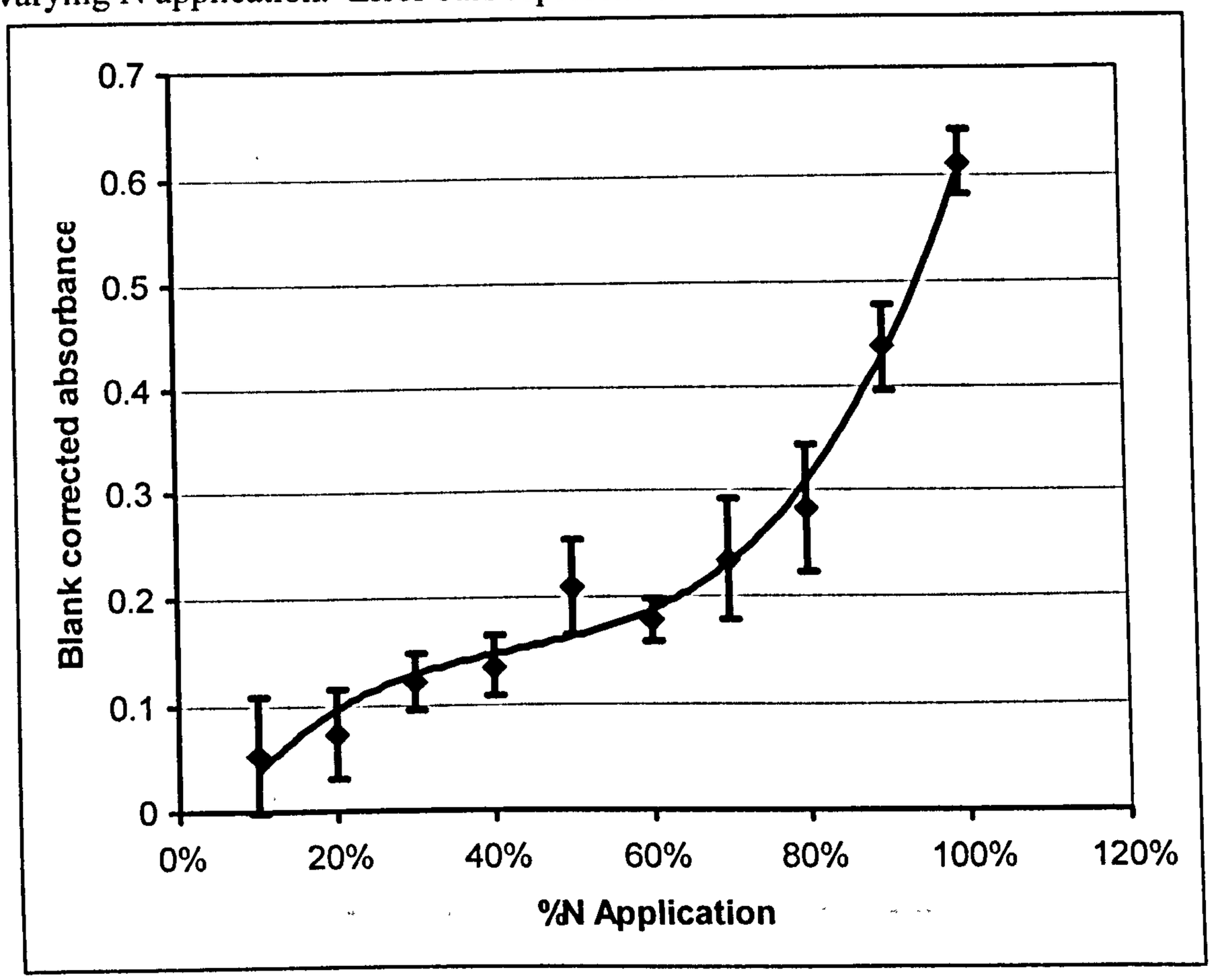

Figure 2.5 – Bradford assay - Relationship between blank corrected absorbance at 595nm and the %N application to the parent plant. Error bars represent one standard deviation.

The response of extractable, soluble, leaf protein (represented as the blank adjusted absorbance at 595nm) to %N application was also non-linear (see figure 2.5). A third order polynomial equation was fitted to the data. The equation of best fit, with an R^2 value of 0.9854, was:

$$y = 1.9529x^3 - 2.4896x^2 + 1.2035x + 0.1784$$

Where y = the blank corrected absorbance at 595nm and x = the %N application to the parent plant.

Since samples were matched, the correlation between the blank corrected absorbance at 595nm and the %N per leaf was also determined (see figure 2.6). A linear model was fitted to the data. The equation of best fit, with an R^2 of 0.908, was:

$$y = 8.4798x + 0.4802$$

Where y is the % leaf N and x is the blank corrected absorbance at 595nm.

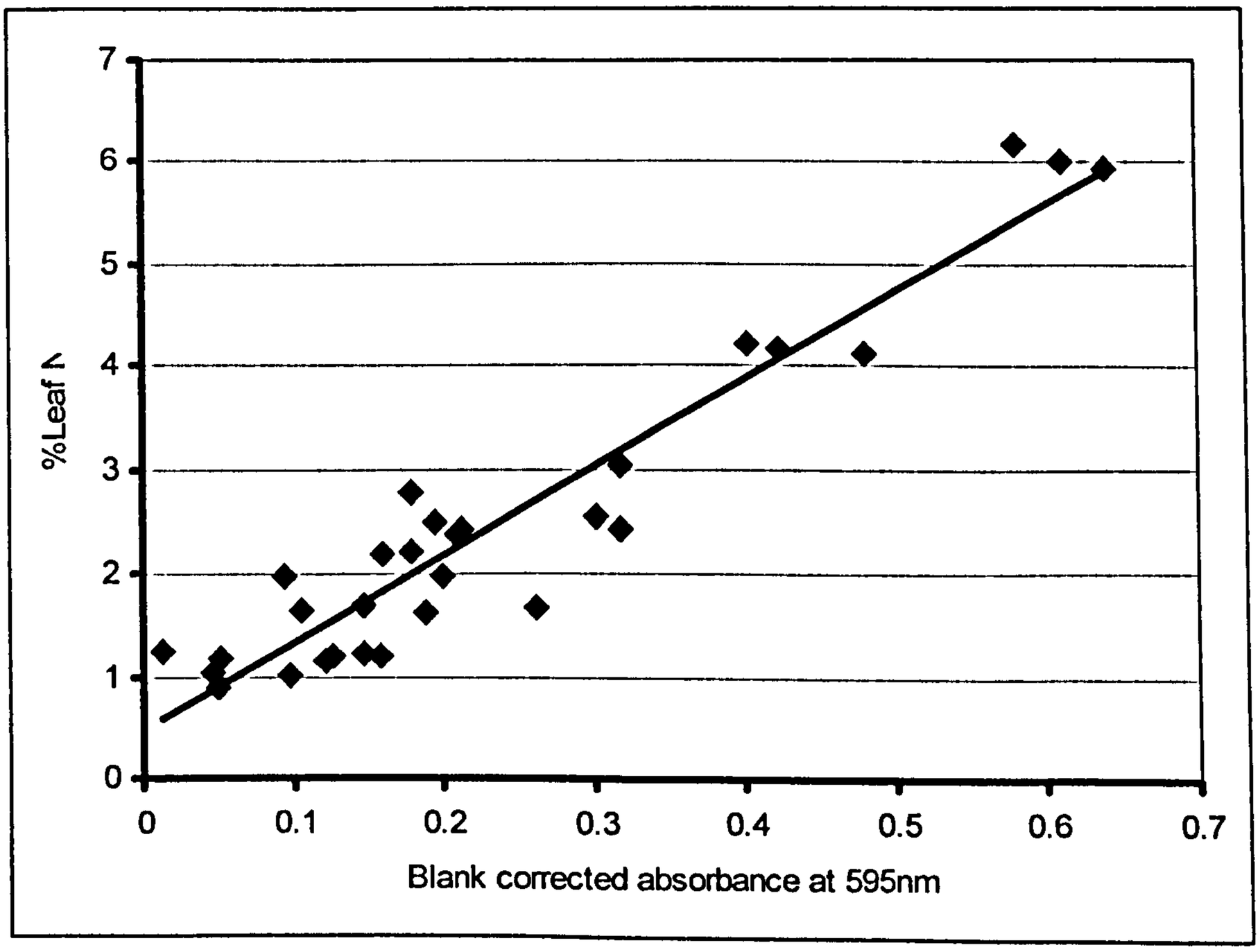

Figure 2.6 – Relationship between %leaf N and blank corrected absorbance at 595nm.

2.5.4. Discussion

Both methods of analysis resulted in a third order polynomial relationship between %N applied to the plant and the measured response. This response pattern could be the physiological response to varying %N application or the result of some artefact of the experiment. Provided this response pattern is repeatable then it does not pose a threat to the methodology.

The two methods used in the experiment measure two different, though related, things. If large globular proteins make a significant proportion of the protein in the leaf then the % leaf N would appear higher than a measurement of the soluble protein by the Bradford method would suggest. However, provided that the composition of proteins does not significantly vary with the N applied to the plant, then the linear model applied to the correlation between % leaf N and the blank corrected absorbance at 595nm should apply.

Plant protein and % leaf N can be manipulated using variably N application. This can be satisfactorily measured using the Bradford assay standardised to % leaf N as determined by LECO C-N analysis.

2.6. VLA Experiment 2: Comparison of oviposition rates on strawberry with variable N applications under conditions of choice and absence of choice

2.6.1. Method

In 1998 a second experiment was performed. Fifty strawberry plants (cv Red Gauntlet) were potted in 500µm sieved sand into 15cm pots. The plants were then watered daily with distilled H_20 for two weeks to wash away any remaining nutrients. Plants were then randomly allotted to a treatment regime of 100%, 75%, 50%, 25% or 0% N. The plants

were fed the nutrient regimes for the entirety of the experiment and two weeks prior to the experiment in the manner previously described. After two weeks the plants were allotted to choice or non-choice treatments. Condition and treatments of host plants were matched between conditions of choice and non-choice to enable a paired t-test to be used for analysis.

The choice treatments were placed in the VLA in a Latin square formation and the non-choice were placed in the individual enclosures previously described. Fifty adult vine weevil were then placed in the VLA and five adults placed in each of the individual enclosures (pitfall traps and direct searching of the VLA prior to the experiment failed to find any overwintering or recently eclosed adult vine weevil).

Collection of substrate to investigate for the presence of ova began in the first week of May 1998 and concluded thirteen weeks later. The top 2.5cm of substrate from around each plant was then replaced every week and the removed sand analysed for the presence of ova as previously described.

Leaf samples, in the form of 3x5mm discs, removed from the leaf margin of three separate leaves of each plant were removed for analysis at the same time as the substrate samples were taken. The leaf samples were kept at minus 70^{o}C until the end of the experiment. At the end of the experiment the leaf samples were prepared and analysed as previously described. Each microplate of 96 wells contained four ultra pure water blank controls and four standard BSA confounding controls.

2.6.2. Transformation of data

The optical density readings (OD) at 595nm derived from the Bradford assay for each plate were blank corrected by subtracting the mean OD reading for the blank controls. A

confounding error ratio was calculated by dividing the mean (blank corrected) BSA standard control reading for the plate by the mean (blank corrected) BSA standard control reading for the control plate (which was used to create the correlation linear model between blank corrected OD and %leaf N). The blank corrected OD readings were then corrected for confounding errors by multiplying the blank corrected OD by the confounding error ratio. This adjusted figure was then transformed into % leaf N using the previously derived linear model.

A general linear model ANOVA was performed with (calculated) asin transformed % leaf N as the response and week, treatment and choice as crossed factors in the model. A second GLM ANOVA was applied to oviposition as the response and week, treatment and choice as crossed factors in the model. A paired t-test, comparing conditions of choice to conditions of non-choice was also performed.

2.6.3. Results

The analysis of asin transformed % leaf N as the response and week, treatment and choice as crossed factors, revealed that there was not a significant difference attributable to choice (paired t-test: $F=0.01$, $P=0.906$) or to week (GLM ANOVA: $df=12$, $F=0.49$, $P=0.918$). Treatment, however, did yield a significant result (GLM ANOVA: $df=4$, $F=769.73$, $P<0.001$) – (see Figure 2.7 and Figure 2.8).

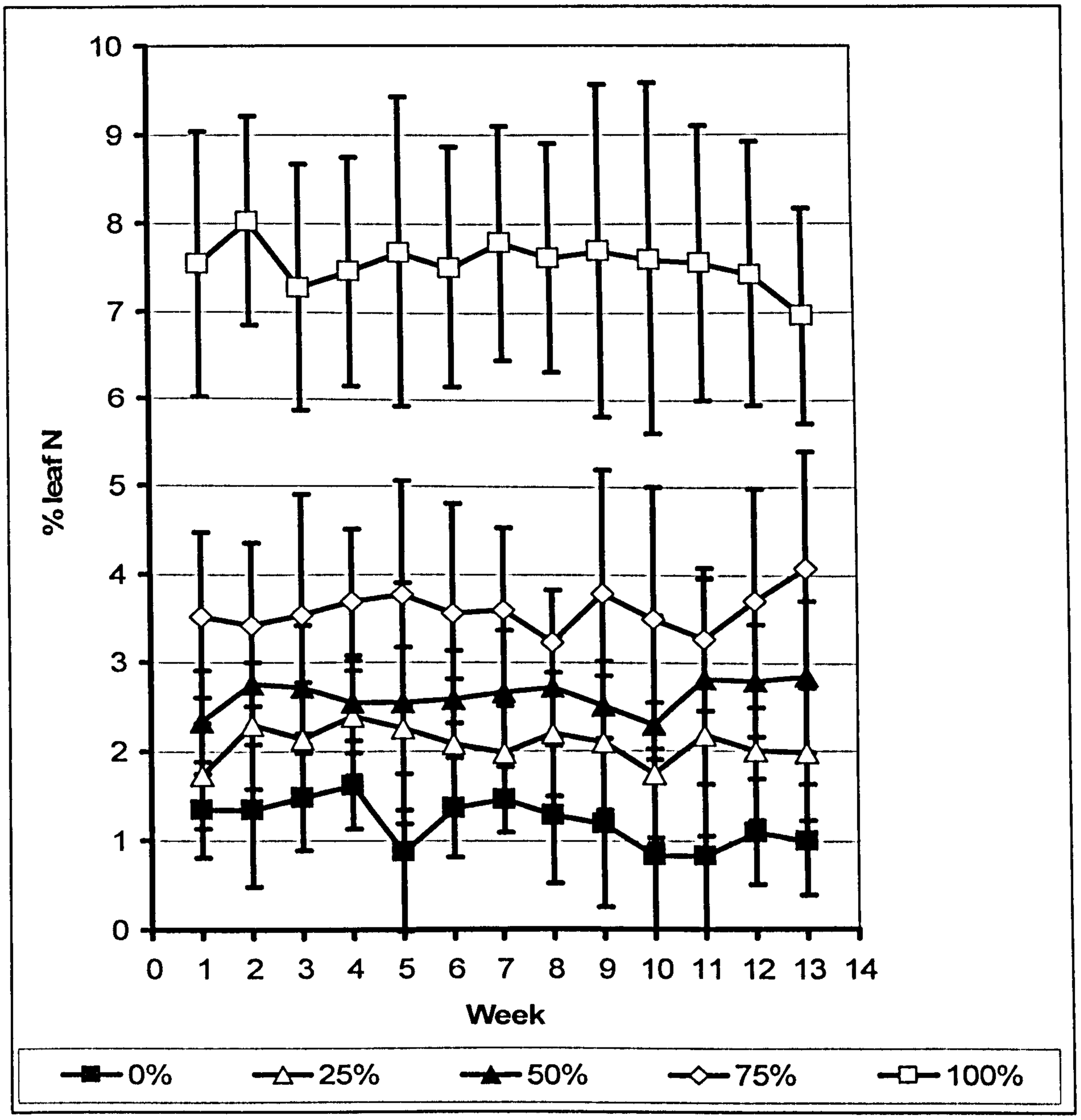

Figure 2.7 – Mean calculated % leaf N per treatment per week. Error bars represent one standard deviation.

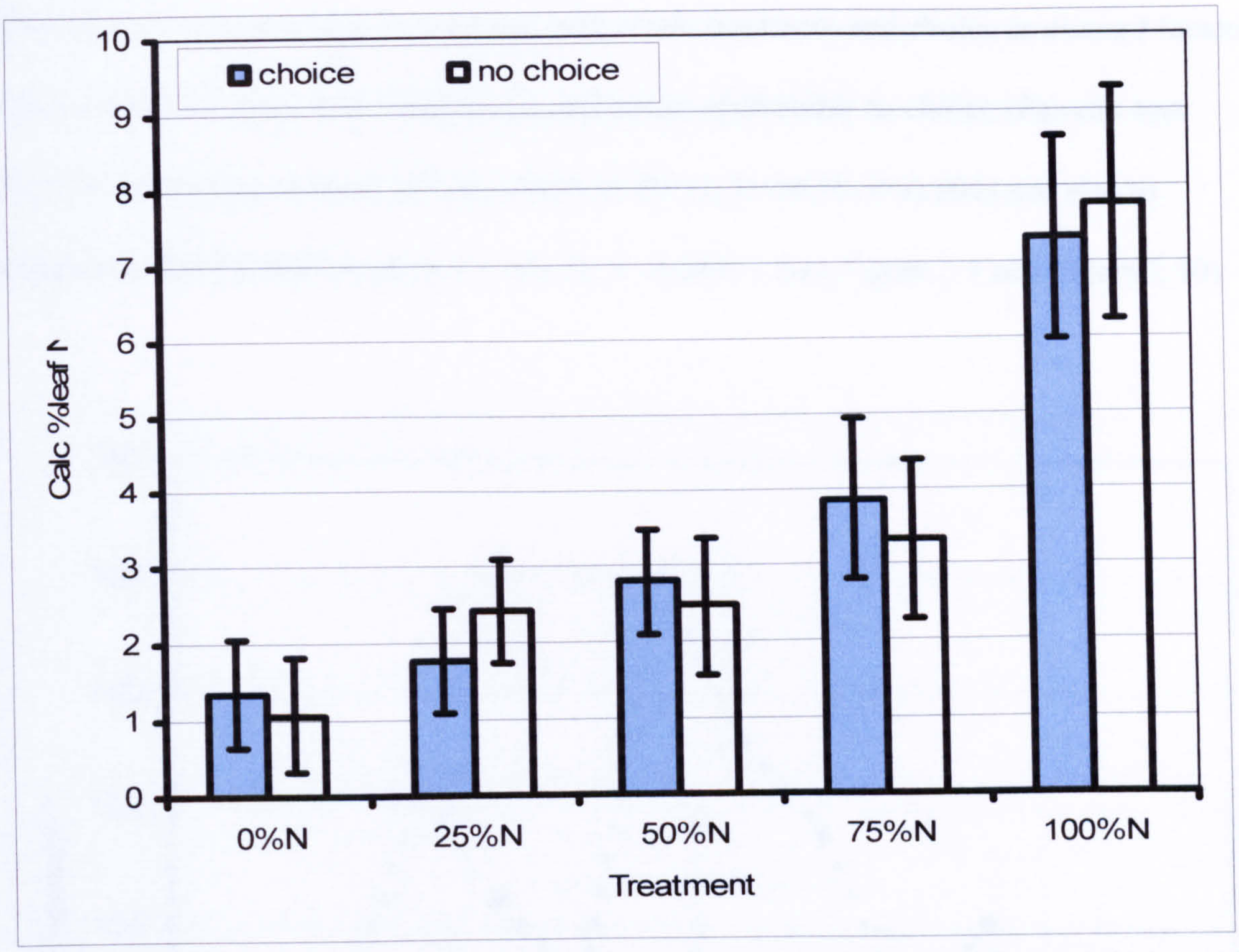

Figure 2.8 – Effect of treatment upon calculated % leaf N under conditions of choice and non-choice. Error bars represent one standard deviation.

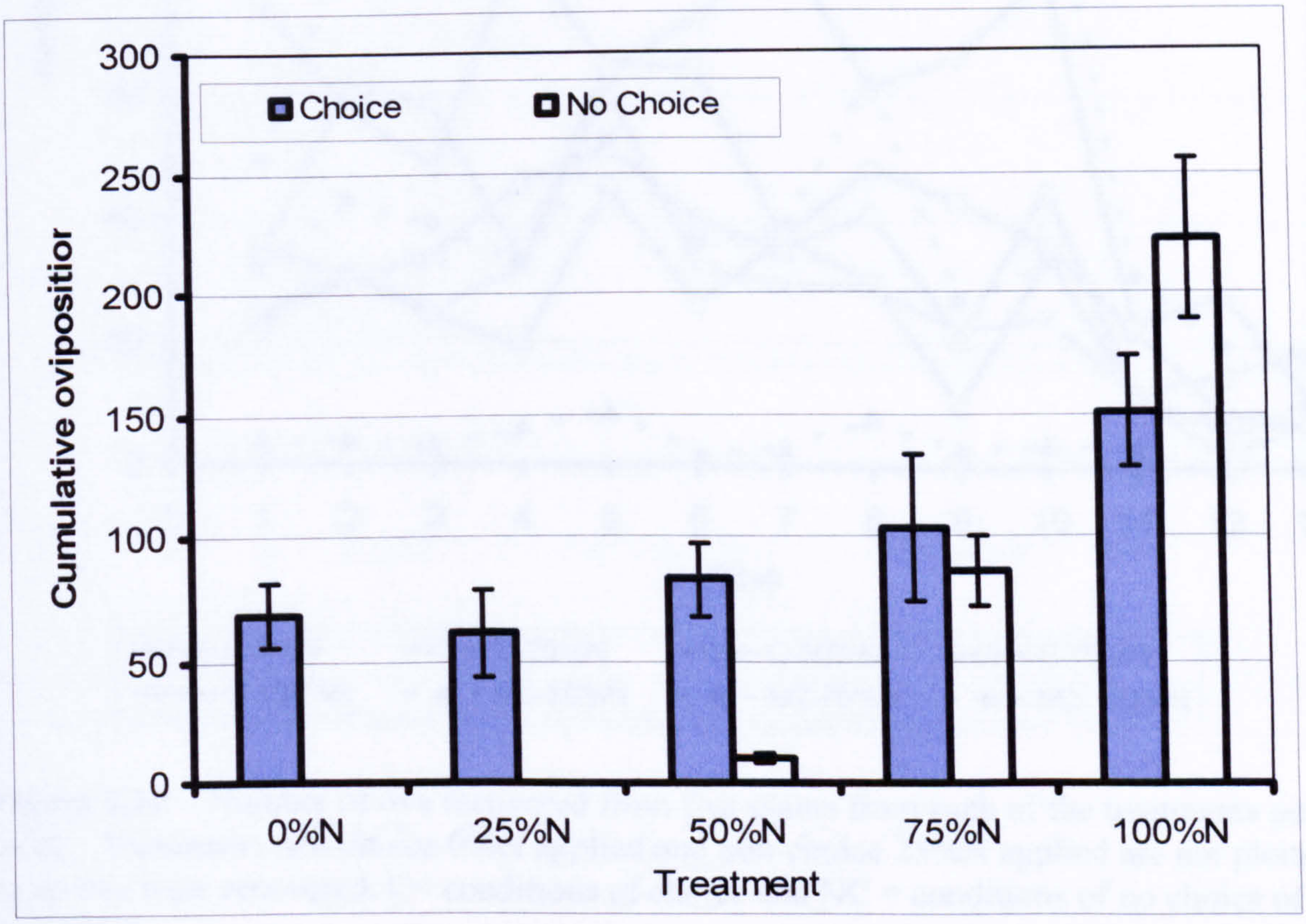

Figure 2.9 – Cumulative oviposition with varying application of N to the host under conditions of choice and non-choice of oviposition host. Error bars represent one standard deviation.

The analysis of oviposition as response with week, treatment and choice as crossed factors, revealed that the there was a significant difference attributable to choice (Paired t-test: $F=27.93$, $P<0.001$), to week (GLM ANOVA: $df=12$, $F=10.39$, $P<0.001$) and also to treatment (GLM ANOVA: $df=4$, $F=109.71$, $P<0.001$) – (see Figure 2.9 and Figure 2.10).

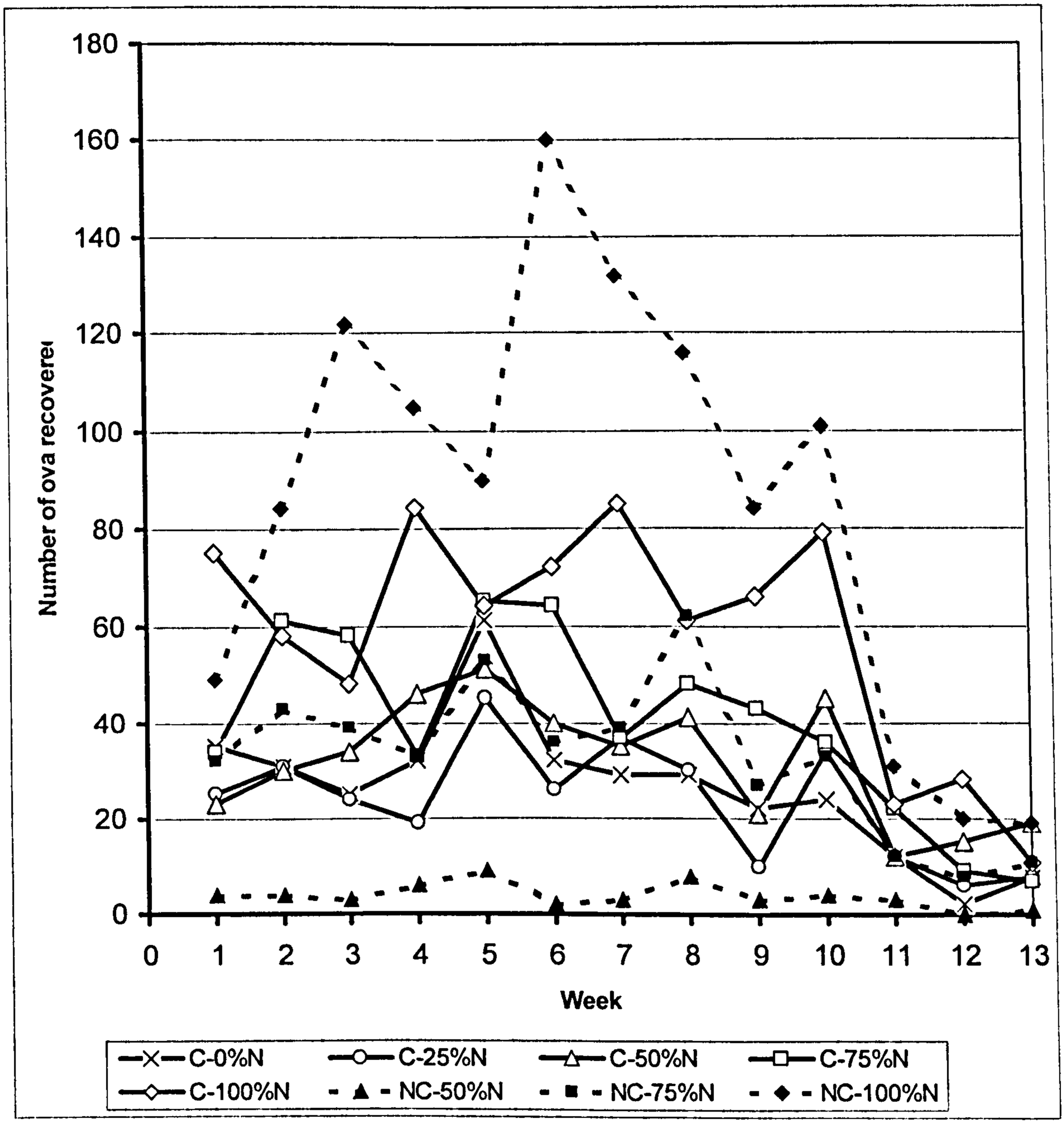

Figure 2.10 – Number of ova recovered from five plants from each of the treatments per week. Treatments non-choice 0%N applied and non-choice 25%N applied are not plotted as no ova were recovered. C= conditions of choice and NC = conditions of no choice of host plant.

The ratio of ova recovered to the (calculated) % leaf N for each plant and week was determined. The paired t-test, comparing conditions of choice to conditions of non-choice, revealed a significant difference (t=3.28, P=0.001) between the ratios for the two treatments (see Figure 2.11).

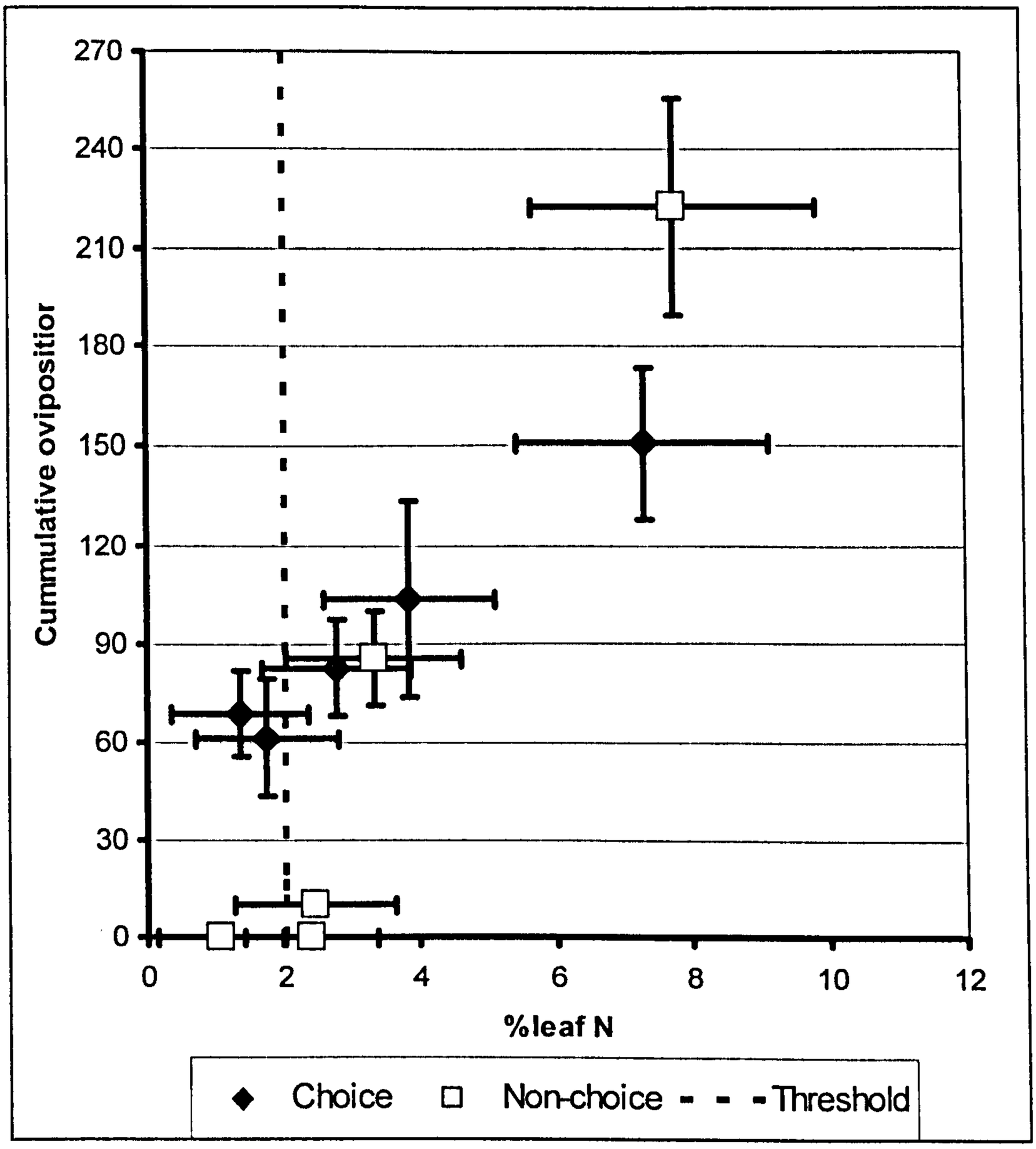

Figure 2.11 – The effect of (calculated) % leaf N upon cumulative oviposition under conditions of choice and non-choice. Error bars represent one standard deviation. Dotted line represents the 2% threshold to oviposition of Hesjedal (1984)

2.6.4. Discussion

The first observation is that the nutritional quality of the host plays less of a role under conditions of choice than under conditions of absence of choice. This may be a response to the duality of purpose of the host to the vine weevil. That is, the host must fulfil the nutritional need for the creation of ova as well as the nutritional need of the larvae for growth and development.

The quality of the leaves of a host plant is likely to correlate with the quality of the roots of the plant since each is inextricably linked to the function of the plant. The function of the leaves cannot be sustained without sufficient root structure and the function of the root system cannot be sustained without sufficient leaf structure. The quality of the leaves consumed by the adult should, therefore, provide a suitable indicator of the suitability of the host as an oviposition site.

It would be expected, therefore, that the level of oviposition should be proportional to a combination of the leaf matter consumed and the nutritional quality of the leaf matter consumed. Thus, if feeding site choice and oviposition site choice were synonymous, then oviposition would not occur upon hosts that could not support the production of ova by the adult. Thus if the % leaf N of any potential host plant falls below the 2% threshold postulated by Hesjedal (1984) then oviposition should not occur. This is not the case under conditions of choice of oviposition site. The conclusion, therefore, is that feeding site choice and oviposition site choice are not synonymous. Feeding and oviposition are independent.

The second observation is that, with the exception of 100%N application, total oviposition under conditions of choice is greater than total oviposition under conditions of absence of choice for each. In addition, cumulative oviposition, for all treatments under conditions of

choice, is greater than under conditions of an absence of choice (2332 choice, 1591 non-choice). This is despite the fact that the effective population density per plant was more than double for the non-choice plants (5 per plant for the non-choice plants and 2 for the choice plants).

The differential in cumulative oviposition between choice and non choice reinforces the "star-player" hypothesis previously described. Even though the number of adults per plant is higher, under conditions of absence of choice, only 1 in five of the vine weevil adults has access to each of the five host types. Under conditions of choice, however, all 50 adults in the VLA have equality of access to each of the host plants. Thus, where the production of eggs is proportional to the host nutritional quality then, in the absence of choice, the cumulative probability of producing ova is reduced. Given the duration of the trial the "star player" hypothesis could not be solely attributed to the cyclical oviposition postulated by Penman and Scott (1976).

The number of adults in the VLA before the experiment began was assessed to be zero. However, if oviposition occurred onto the underlying substrate of the VLA and there was sufficient "weed" roots in the substrate to allow the full cycle of development it is possible for the adult population of the VLA to be supplemented by recruitment. For this to explain the entirety of the disparity between choice and non-choice trials an extimated134 vine weevil adults would have to have been recruited in the VLA at the very beginning of the trial. The probable level of recruitment is much less, if any, than the 134 required. The disparity, therefore, is more likely to be a product of the star player hypothesis.

The third observation is that the seasonality of oviposition is not a response to changes in host nutritional quality. Oviposition was found to be seasonal - % leaf N was found not to be seasonal.

Chapter 3. - Tracking of *Otiorhynchus sulcatus* movement during oviposition and host choice within a large arena using passive transponders

3.1. Introduction

Phylogenetic constraints (Pires *et al,* 2000) or neural constraints on information processing (Janz and Nylin, 1997) should generally lead to plant-feeding insects to become relative specialists. This hypothesis predicts that this neural limitation will result in a cost being paid either; in increased decision/association time with the host plant, or in accuracy of matching oviposition to host suitability (Janz, 2003; Nylin *et al*, 2000; Janz & Nylin, 1997).

Optimal oviposition theory suggests that oviposition will occur upon plants which the larvae will perform best (Steinbauer, 1999). However, under conditions where the host plant is also the adult feeding site, behaviour may favour adult preferences at the expense of larval suitability (Schiers, 2000; Schiers *et al*, 2002).

Marginal Value Theorem (Charnov, 1976) predicts that a forager should leave a patch when the instantaneous capture rate has fallen to the average capture rate for the habitat (Alonso *et al*, 1995).

Does the level of oviposition vary according to the quality of the host? If so; is it a purely physiological conversion factor? A high quality host, in terms of quality of leaves for supply of nutrients to the adult, would be expected to have a high quality root system for the supply of nutrients to the larval vine weevil. A purely physiological strategy would be represented by a system where there is a direct transformation of the nutrients derived from the host plant to the production of ova, which are then oviposited at the same host site.

This strategy would be characterised by low mobility between feeding and oviposition events, low levels of discrimination between host plants with respect to residency time, a high correlation between feeding and oviposition and the absence of, or minimal, storage of ova.

An alternative strategy would be a purely Marginal Value Theorem (Charnov, 1976) behavioural response. Sampling or other investigations of the host by the adult weevil, results in a clear decision to remain on a host to oviposit or to move on to a higher quality host. This strategy would be characterised by host quality related mobility between feeding and oviposition, the potential absence of a relationship between feeding and oviposition, and the ability to retain ova, to delay oviposition.

The results of the previous experiments, concerning oviposition under conditions of choice, could not be linked to the specific activities of individual vine weevil. The purpose of this experiment, therefore, was to determine the relationship between feeding and oviposition and the specific activity of individual vine weevil adults under conditions of choice of oviposition host.

3.2. Methodology

The results of the previous experiments were skewed by the effect of seasonality. It was decided, therefore, to conduct the experiment within an indoor arena under controlled lighting conditions. Lighting was provided by four 1,000W ventilated mercury vapour lamps on a 16:8 day/night cycle.

The design of the experiment followed a simple 4 x 4 grid of strawberry plants (cv Red Gauntlet). The experiment was conducted within a 2m x 2m indoor arena sealed at the base and secured using a 4cm strip of petroleum jelly around the top of the arena wall (see

figure 3.1). Two variables were introduced in the form of; amount of nutrients applied to the host plants and the presence or absence of a surface refuge covering. Visits to host plants by a vine weevil were determined using passive transponders. Oviposition, leaf consumption and leaf samples for the determination of % leaf N were taken.

The experiment was set up and the adult vine weevil introduced to the arena in the first week of May 2000. Substrate and leaf sampling began one week later.

Figure 3.1 – Experimental set up of the indoor arena showing the four by four grid pattern of strawberry plants and surface coverings.

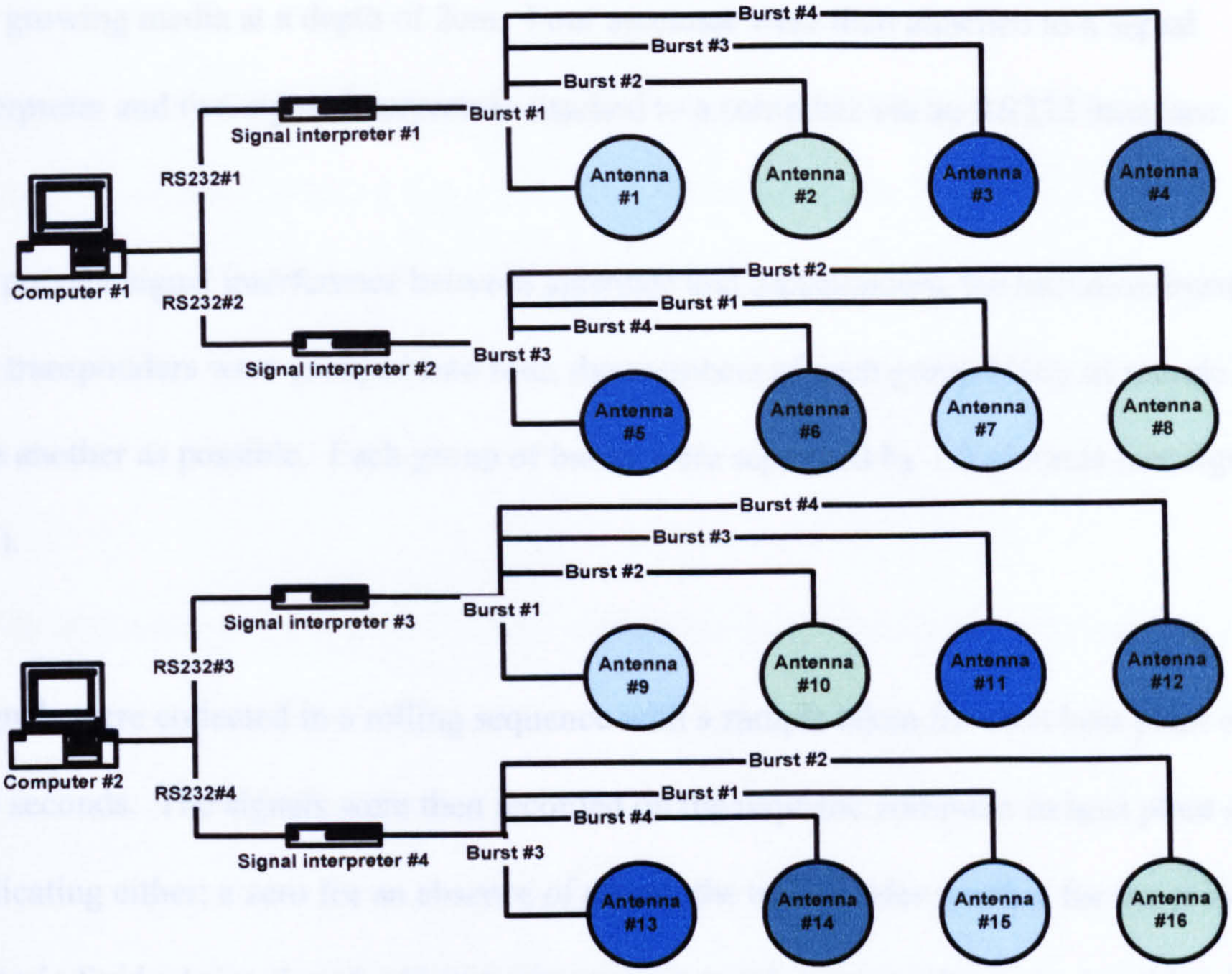

Figure 3.2 – Antennae set up and burst sequence – same colour denotes same burst sequence.

3.2.1. Passive transponders

Each of the fifteen adults used in the experiment had a small passive transponder glued to its back. The transponders used in the experiment were Texas Instruments HDX (half-duplex) glass encased micro-transponders operating at 123.2 KHz. For the purpose of the experiment the transponders were removed from the glass casing to reduce the size and weight. The transponders were then tested, applied to the back of a vine weevil using super glue, and then tested again. The range of the detectable signal was found to be 25-40mm (depending upon transponder and plane of orientation to the receiving antenna).

Signal induction of the passive transponders and return signal capture was performed by simple 15cm diameter laminar antennae, with a 2cm diameter hole in the centre, and with inbuilt signal amplification chip. Each host plant was equipped with an antenna buried in

the growing media at a depth of 2cm. Four antennae were then attached to a signal interpreter and two signal interpreters attached to a computer via an RS232 interface.

To prevent signal interference between antennae and transponders, the initiation burst for the transponders were grouped into four, the members of each group being as remote from one another as possible. Each group of bursts were separated by 1.5 seconds (see figure 3.2).

Signals were collected in a rolling sequence with a sample taken for each host plant every 7.5 seconds. The signals were then recorded on the requisite computer in host plant order indicating either; a zero for an absence of signal, the transponder number for the presence of an individual signal, or "col" (meaning collision) where more than one signal has been received and the specific signals cannot be distinguished. Due to software constraints the results were then collated into; specific vine weevil present, absence of signal, and collision, in fifteen minute intervals.

3.2.2. Preparation of host plants

Host plants were potted into 16cm diameter pots using 500μm sieved sand as the growing media. The pots were sealed except for the drain tube at the base. Each pot also contained an antenna, an automated feeding device (calibrated to 120ml per day) and a drench/drain tube. The plants were then randomly separated into two groups and fed miracle-gro®, at either 100% or 10% manufacturers recommended concentration, for two weeks prior to the experiment commencing. Nutrient solutions were supplied at 120ml per day via the automated feeding system with two 2.5 litre drench and drain treatments applied once per week to remove extraneous nutrients. During the experiment plants were gravity fed from nutrient reservoirs connected via tubes passed through holes drilled through the floor of the arena.

During the experiment the surface of four of the high nutrient and four of the low nutrient plants were covered in 2cm x 2cm squares of grey masking cloth to act as a refuge. The plants were then arranged such that each row and each column contained one of the four treatments (HR, LR, HnR, LnR – H = high nutrient, L = low nutrient, R = refuge, nR = no refuge).

At the beginning of the experiment each trifoliation on each plant was labelled with a small wax paper ring at the base of the leaf. Leaves that formed during the experiment were also labelled and scanned. Any runners that formed during the experiment were excised.

3.2.3. Extraction of ova

The top two centimetres of substrate were collected daily in the last half hour of "daylight" and the ova extracted as previously described.

3.2.4. Leaf scanning for determination of feeding

Leaves were scanned by placing the leaf between a piece of hardboard and a thin sheet of glass and then the image captured in silhouette format using a hand scanner. The area of feeding was estimated by comparing a scan of the leaf containing leaf notches with the previous scan of the same leaf. The two scans are aligned, the periphery of the leaf before feeding occurred determined, and the area of feeding calculated (see figure 3.3).

All leaves were scanned at the beginning of the experiment, once per week during the experiment, and daily if any feeding notches were apparent.

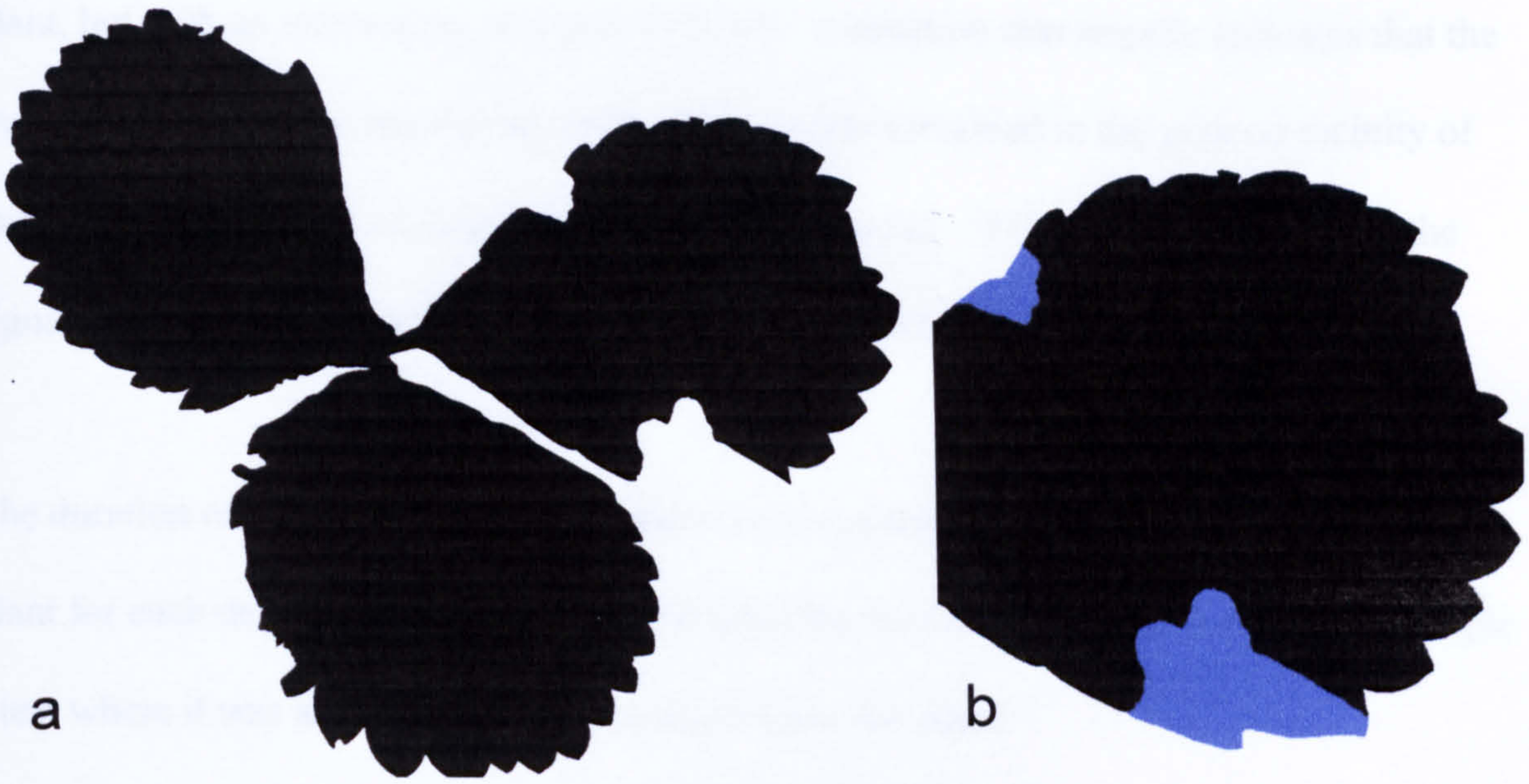

Figure 3.3 – a Strawberry trifoliation - one leaf with vine weevil feeding notches and **b** the same leaf with the area of the leaf before feeding replaced in blue.

3.2.5. Leaf sampling for measurement of N and protein content

Three 5mm diameter disks were removed from the periphery of three leaves from three different trifoliations of every plant every week. Samples of whole leaves were taken at the end of the experiment. The samples will be kept at -20°C pending analysis.

3.2.6. Analysis of data

Paired t-tests were used to compare high nutrient with low nutrient and refuge with no refuge. The correlation between feeding and oviposition was compared using Pearson. One way ANOVA was used to determine whether oviposition or feeding was found to be seasonal.

In order to analyse and relate the transponder data to oviposition and feeding the data was categorised into three behaviours; "R", "C", and "H" based upon consecutive transponder readings. "R" was indicated when the consecutive signals received indicate movement to another plant. "C" was indicated when the following signal received was for the same

plant, but with an intervening absence of signal – a situation that usually indicates that the weevil has moved into the canopy of the plant, or has remained in the general vicinity of the same plant, but out of range of the transponder signal. "H" was indicated where the signal was persistently in the same location without intervening blank space.

The duration of each activity was determined and expressed as a cumulative total for each plant for each day. The data were then analysed for the two treatments using a two sample t-test where it was assumed that the variances were not equal.

The period of time with which the vine weevil adults remained in association with the host plant – expressed as the period of time between the first transponder signal and the last transponder signal, without an intervening signal from another plant, was determined. This data was then analysed using a two sample t-test where it was assumed that the variances were not equal.

3.3. Results

3.3.1. Effect of N application upon feeding and oviposition

The level of oviposition was not significantly different for the two N treatments (paired t-test t=-1.46, P=0.147). Oviposition was also found not to be significantly different (paired t-test t=-1.47, P=0.145) when transformed into weekly probability of an oviposition event occurring. The data does not support a hypothesis that oviposition is responsive to nutritional quality of the host plant.

The amount of leaf area consumed was significantly different between the two treatments (paired t-test t= 2.06, P=0.043). The probability of a feeding event occurring in a given week, however, was found to be not significant as all plants had some feeding plants of

some sort for every measurement period. The data suggests an equality of probability of feeding occurring with a greater proportion of leaf material being consumed from plants of a higher nutritional value.

3.3.2. The effect of refuge upon oviposition and feeding

The level of oviposition is significantly different between hosts with a refuge compared with hosts without refuge (paired t-test t= 2.77, P=0.007). The probability of an oviposition event occurring, however, was found not to be significant (t=4.65, P<0.001). The data appear to suggest that the presence of a refuge does not increase the probability of oviposition occurring but does increase the number of eggs oviposited at a site.

Surprisingly, the amount of leaf consumed was also found to be significantly different between the two treatments (t= 3.78, P<0.001). The probability of a feeding event occurring was found to not be significant as feeding occurred upon all plants between measurements (probability of a feeding event = 100% for all plants). Refuge therefore increases the amount of feeding that occurs.

3.3.3. Correlation between feeding and oviposition

Pearson correlation relating oviposition to feeding for each of the four treatments was conducted (see table 3.1).

Treatment	Probability of oviposition Pearson	P	Quantity of oviposition Pearson	P
100% Nutrient	-0.099	0.759	-0.008	0.837
10% Nutrient	0.368	0.239	0.074	0.054
Refuge	0.227	0.478	-0.009	0.824
No refuge	0.063	0.551	0.062	0.11

Table 3.1 – Pearson correlation relating oviposition and feeding under conditions of varying nutrient application and presence or absence of surface refuge.

The analysis suggests that feeding and oviposition are not correlated for any of the treatment regimes, whether based upon a direct comparison of quantity, or based upon the probability of an event occurring.

3.3.4. Seasonality

Oviposition is not seasonal (one way ANOVA F= 1.46, df= 11, P=0.149). Figure 3.4 does reveal a reduction in oviposition at the beginning of the experiment – this could be attributed to transition to experimental conditions rather than true seasonality. Feeding is also not seasonal (one way ANOVA F=1.29, df= 11, P=0.235) see Figure 3.5.

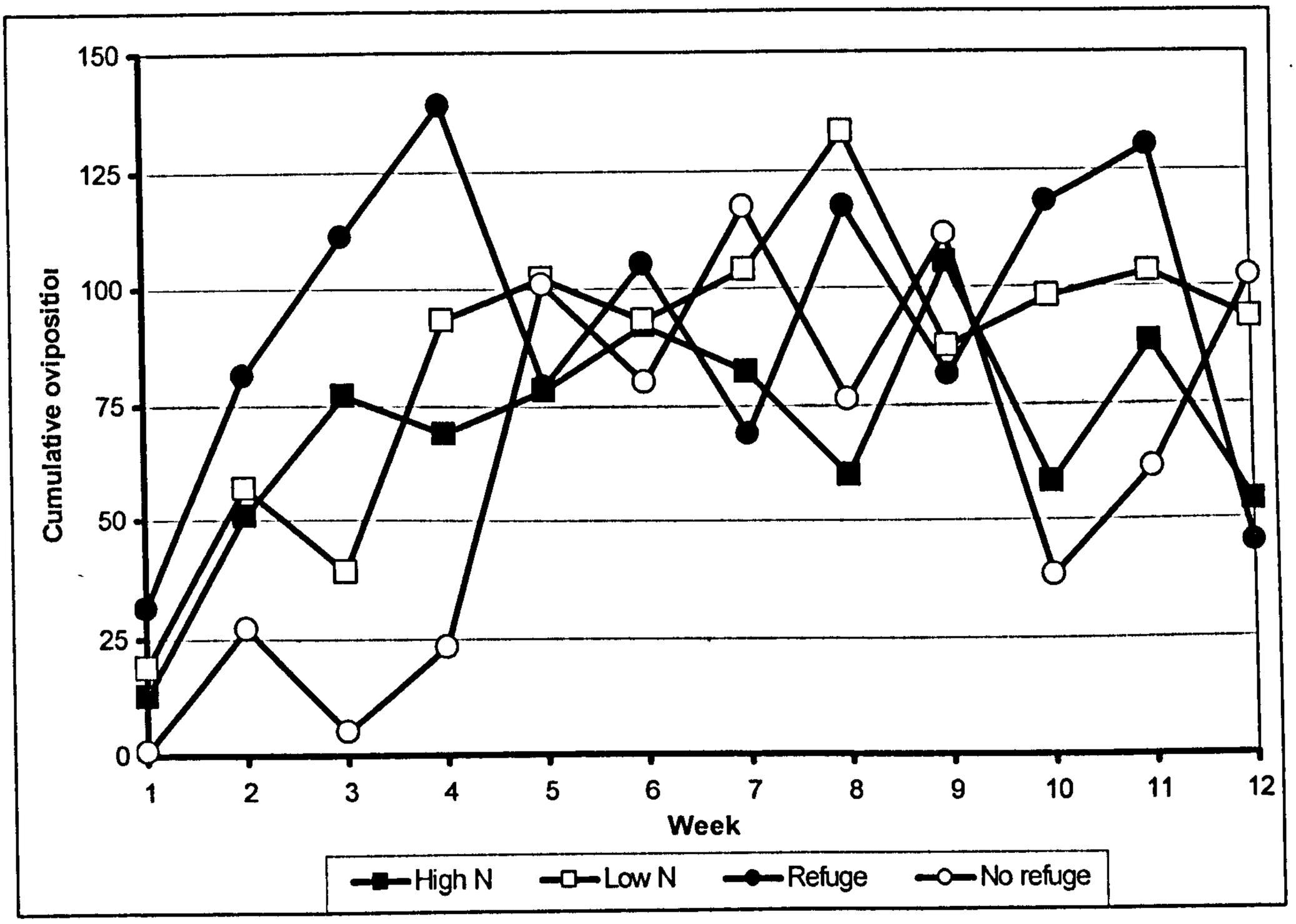

Figure 3.4 – Oviposition by treatment by week.

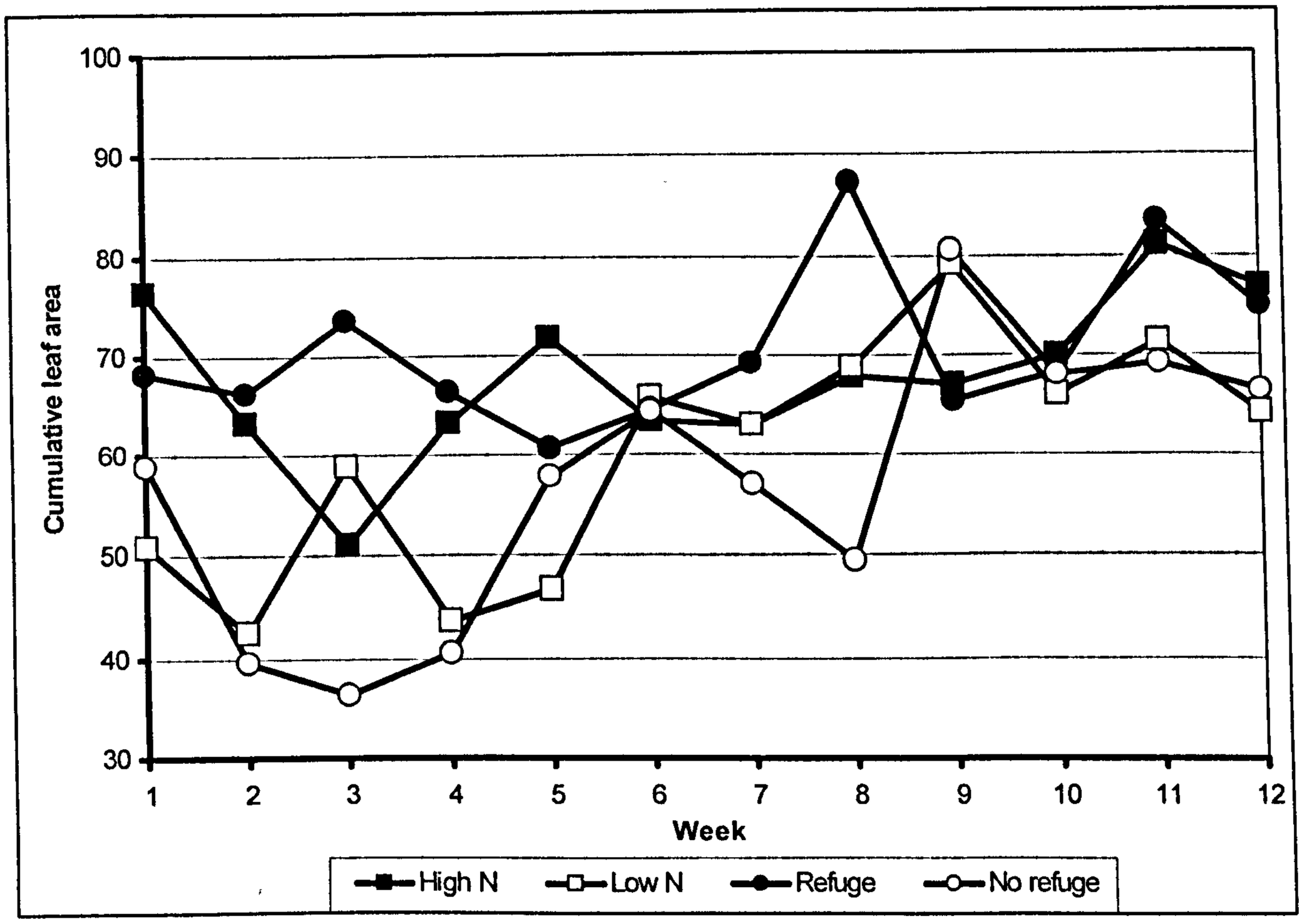

Figure 3.7 – Leaf area consumed (mm^2) by treatment by week.

3.3.5. Protein content

Samples recovered from the experiment have yet to be analysed.

3.3.6. Behaviour - Vine weevil host plant visits and oviposition

Since the number of vine weevil adults recorded as visiting the respective plant on the day of an oviposition event can be greater than one, the relative number of transponder signals for each weevil, expressed as a total of clear transponder signals, was used. This function, expressed as a probability, gives some idea as to the likely contributor of the ova found at the site.

Only 2 of the 232 oviposition events could be attributed to a single vine weevil visiting the plant on the requisite day. All other oviposition events were characterised by either; the presence of discrete sequential signals from more than one weevil, or by "col" signals, implying that more than one weevil as present at the time of the signal. The vast majority of oviposition events, therefore, are either the product of more than one vine weevil adult or single vine weevil adults undeterred from ovipositing by the presence of other weevils.

3.3.7. Behaviour - Vine weevil host plant visits and behaviour

Variable nutrient supply to the host plant was found not to significantly affect the duration of any of the behaviours (paired t-test: "C" $t=1.47$, $P=0.143$, "H" $t= -1.34$, $P=0.182$, "R" $t=-1.20$, $P=0.229$). The presence of a refuge reduced incidence of movement to an alternative plant (behaviour "R", paired t-test $t=6.54$, $P<0.001$) and increased the length of time spent in association with the host plant (paired t-test "C" $t=11.78$, $P<0.001$ and "H" $t=6.26$, $P<0.001$).

3.3.8. Behaviour - Vine weevil residency behaviour and association time

Association time was found not to be significant for presence of a refuge (two sample t-test df=9, t=-0.00, P=0.996) and also not significant for nutrient supply to the host plant (two sample t-test df=9, t=-0.00, P=0.999). A greater proportion of the visits of greater than 24 hours were found to occur upon host plants with a refuge or with higher nutrient application regime (see Figure 3.8).

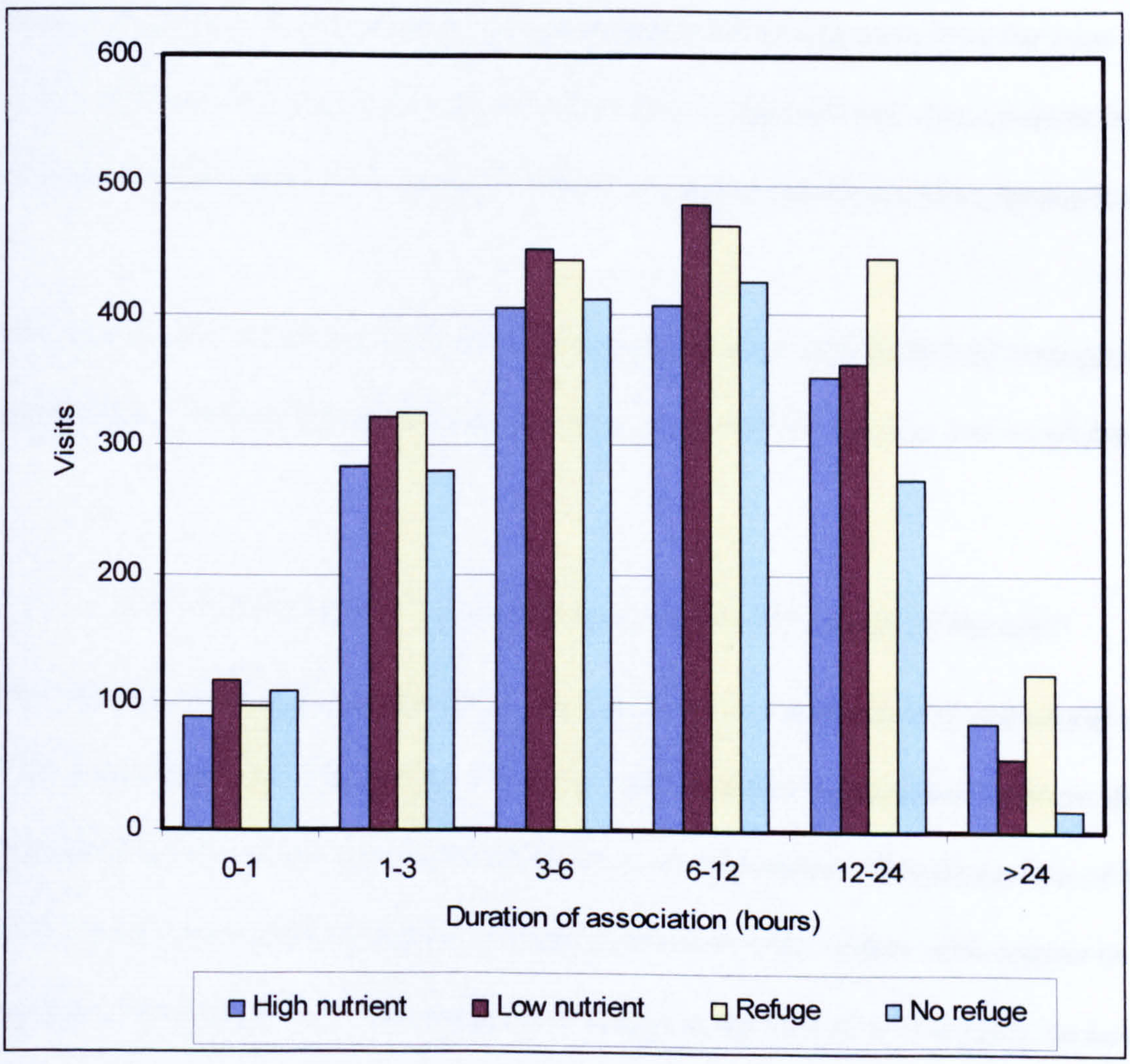

Figure 3.8 – Duration of association time by treatment.

3.4. Discussion

3.4.1. Technological issues

Two major drawbacks of this method of tracking are:

- That you cannot distinguish between adults that enter and leave the vicinity of the host plant from the ones that exceed the ranges of the transponders when they go into the canopy to feed, and

- The inability to distinguish the signals that contribute to a "col" signal.

To combat the first issue it would be more appropriate to confine future experiments of this type to host plants like polyanthus that do not form leaves disparate from the base. Alternatively, it may be possible to develop a light-weight antenna than could be bonded to the leaf and thus enable time in the vicinity of the host plant leaves to be determined.

The second issue could only be resolved using full duplex "anti-collision" transponder technology. Transponder and signal interpreter chips of this type are now available.

3.4.2. Does the level of oviposition vary according to the quality of the host?

The data does not support a hypothesis that oviposition is responsive to nutritional quality of the host plant. The experiments conducted did not find a relationship between the nutritional quality of the host plant and the level of oviposition. Discrimination of host plants, based upon nutrient quality, was also found not to be evident with respect to residency and association. The presence of refuge at the base of a host plant, however, did increase oviposition and also time spent in contact with the host plant substrate.

The analysis suggests that vine weevils are indiscriminate with respect to oviposition site choice at the scale of observation. Increased oviposition appears to occur either:

- As a secondary consequence of the avoidance of predation, or,

- Vine weevil adults display an acute response to relative humidity, or,

- Vine weevil oviposition behaviour anticipates a greater probability of disadvantageous physical conditions under conditions without a surface covering.

Direct observations of oviposition events indicate that oviposition does not occur directly upon the surface of the substrate. Extremes of temperature and humidity are not expected to be greatly different between a nominal surface covering and the absence of a surface covering. It is expected, therefore, that the increased oviposition and feeding associated with refuge treated plants is a consequence of predation avoidance.

Chapter 4. - Arena based video observation of directional bias in the movement and behaviour of *Otiorhynchus sulcatus*.

4.1. Introduction

Cram (1965b) observed that "[*O sulcatus*] wander randomly at night and encounter hosts fortuitously". The method and strategy that the vine weevil employs in order to encounter a host plant will influence pattern and distribution of oviposition. Given the total reliance upon walking by the flightless coleopteran – how random is the walk that the foraging strategy depends upon?

There are a number of arguments for random movement in the literature; from both a theoretical viewpoint and from attempts to interpret empirical data (Bergman *et* al, 2000; Byers, 2001; Morales and Ellner, 2002; Kareiva and Shigesada, 1983; Wu *et al*, 2000).

Other than as a response to a completely random environment it is difficult to determine an evolutionary advantage to randomness. In the absence of a random environment it would be expected that simpler organisms display a restrictive repertoire of behaviours fixed at the individual if not the population level. There is some evidence to support the view of context-specific fixed individual behaviours (Budaev, 1997; Coleman and Wilson, 1998; Iguchi *et al*, 2001; Reale *et al*, 2000).

Developments in biomathematics have shown that very complex outcomes can arise from simple models (May, 1976). It is possible to suggest that the range of behaviours necessary to thrive in a heterogeneous environment could arise from relatively few rules. Evidence for emergence is now available across a wide range of systems (Johnson, 2001).

Stinner and Bacheler (1993) developed a movement model, based upon simple rules, and defined by three parameters: turning angle, direction of turn and step length. This enabled a diverse range of behaviours from a stochastic spiral pattern to restricted random search to straight line escape.

The purpose of the following experiments was to investigate the characteristics of motion in vine weevil and relate the observations to foraging strategy. Casual observation of vine weevil before the experiment suggested a two stage locomotion process of straight line walking interspersed with rotation about the spot. Casual observation also suggested a pronounced bias for anti-clockwise rather than clockwise rotation.

It was decided, therefore, to investigate locomotion in vine weevil by measuring the prevalence of directional bias, path length and angle of rotation. The method adopted for this investigation was arena based video observation.

4.2. Methodology

4.2.1. Light box arena with time lapse video observation

Experiments were conducted in a 1m x 1m x 2m tall light box with an occludable Perspex windowed door 1m above the base. A 5cm wide strip of the walls around the top of the lower 1m of the light box were covered in petroleum jelly as a secondary method of preventing the escape of any vine weevil that may have escape from the experimental arenas held within the light box. Day/night cycle was maintained by uncovering the Perspex window during the day and, to prevent lab lighting intruding into the box, the window was covered at night. When the window was covered light was provided via a 25W incandescent bulb set to a faint glimmer by the use of a dimmer switch.

Observation of movement was made using a low light black and white video camera, suspended within the light box, recording onto standard stock video tape, using a time lapse video recorder at a time compression of 1 in 4.

Individual experiments were conducted within 30cm diameter circular arenas with a 2cm wide layer of petroleum jelly around the edge to prevent escape.

4.2.2. Experiment 1: Pilot study - Investigation of directional bias and angle of rotation.

The motion of an individual vine weevil, within a circular 30cm diameter arena, was captured over a nine hour period on video tape. The video tape was then inspected and the direction and angle of rotation measured using a protractor held against the monitor screen. Measurements were made in five degree intervals. The angle and direction was then recorded. This was repeated six times with six different vine weevil adults until 1000 observations had been made.

4.2.3. Experiment 2: Investigation of directional bias, angle of rotation and path length.

In this experiment 45 vine weevils were observed during fifteen, two day trials, where three arenas were taped simultaneously. The angle of rotation was measured using a protractor held against the monitor. Any interaction with the edge of the arena was logged and any path not interrupted by the edge of the arena estimated to the nearest five millimetres.

The path length of any circumferential movement about the walls of the arena was also determined. If the path was circumferential, any angle of rotation was measured (when feasible) in the same plane of reference as the vine weevil.

Directional bias data was then expressed as a percentage clockwise to anticlockwise rotations for each vine weevil, the percentage was then arcsine transformed and analysed using a paired t-test.

To test for observer bias in the interpretation/collection of angle of rotation data a further paired t-test was performed comparing 0° with 5°, 10° with 15°, and 20° with 25°, etc.

4.3. Results

4.3.1. Experiment 1: Pilot study - Investigation of directional bias and angle of rotation.

The observation of locomotion of vine weevil adults showed a marked tendency towards anti-clockwise rotation. Only three observations of clockwise rotation were made (two at 35° and one at 50°). The clockwise rotations also appeared "clumsy", requiring greater perturbation of the legs and seemingly more leg movements than the anti-clockwise rotations. Of the anti-clockwise rotations, substantive peaks were recorded at 50°, 130° and 185° (see figure 4.1). However, given the nature of the experimental procedure, all measurements must be considered ±10°.

The relationship between the observation and the vine weevil that contributed to the observation was not recorded. The observations did cease, however, before the end of the sixth recording so it is assumed, therefore, that weevils one to five contributed proportionately more observations to the result than number six.

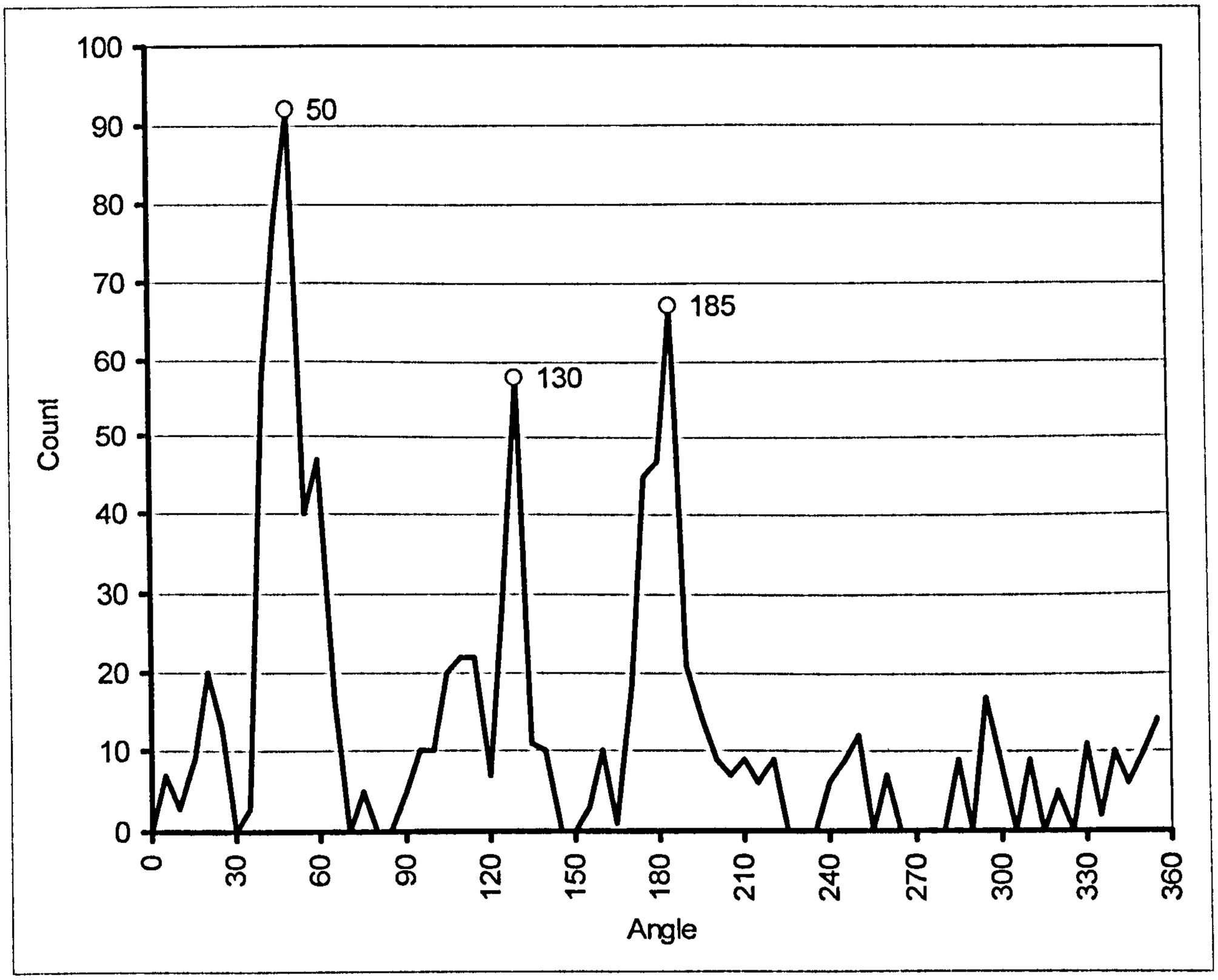

Figure 4.1 – Cumulative frequency of angle of rotation for anti-clockwise rotations. Clockwise rotations omitted. Peaks at 50°, 130° and 185° labelled.

4.3.2. Experiment 2: Investigation of directional bias, angle of rotation and path length.

The results, when collated into a frequency distribution, were skewed towards the shorter rotations - showing a considerable peak at around zero degrees, a marked fluctuation between 10° and 80°, a relative reduction in observations between 80° and 120° followed by a further zone of fluctuation between 120° and 340°, a considerable peak around 360°, with a trailing off of observations towards 420° (see figure 4.2). The pattern of distribution of clockwise rotation is similar to that of anticlockwise rotation but on a reduced scale. The distribution of observation does not closely correlate with the distribution of observations from the first experiment.

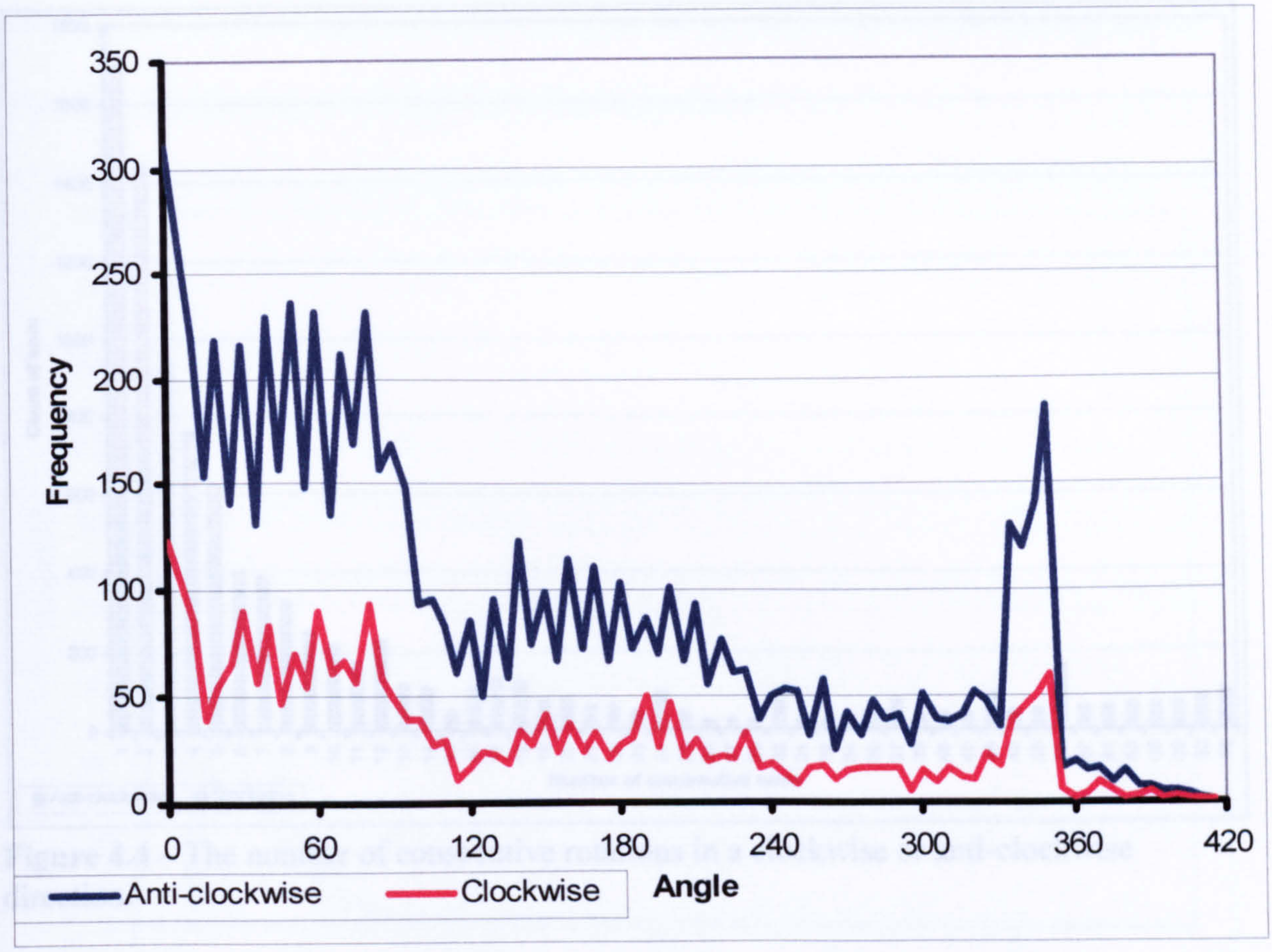

Figure 4.2 – Relative frequency of angle of rotation clockwise and anti-clockwise.

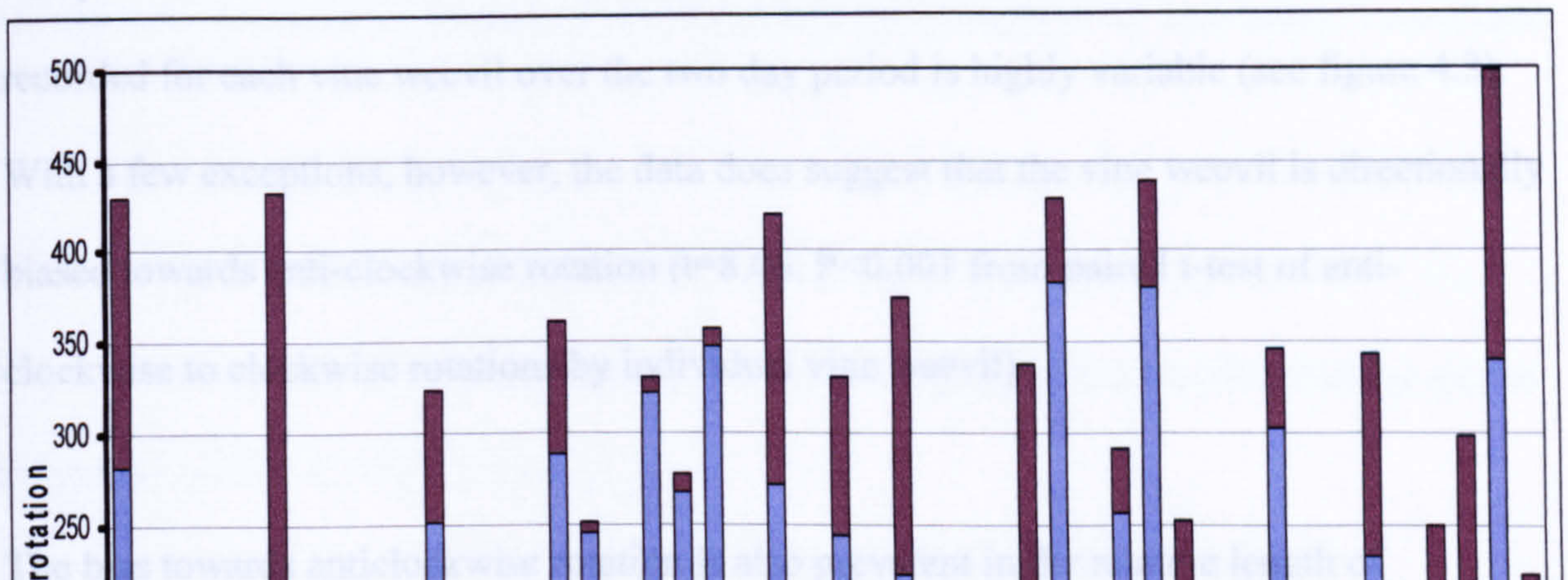

Figure 4.3 – Relative number and direction of rotation of 45 vine weevil.

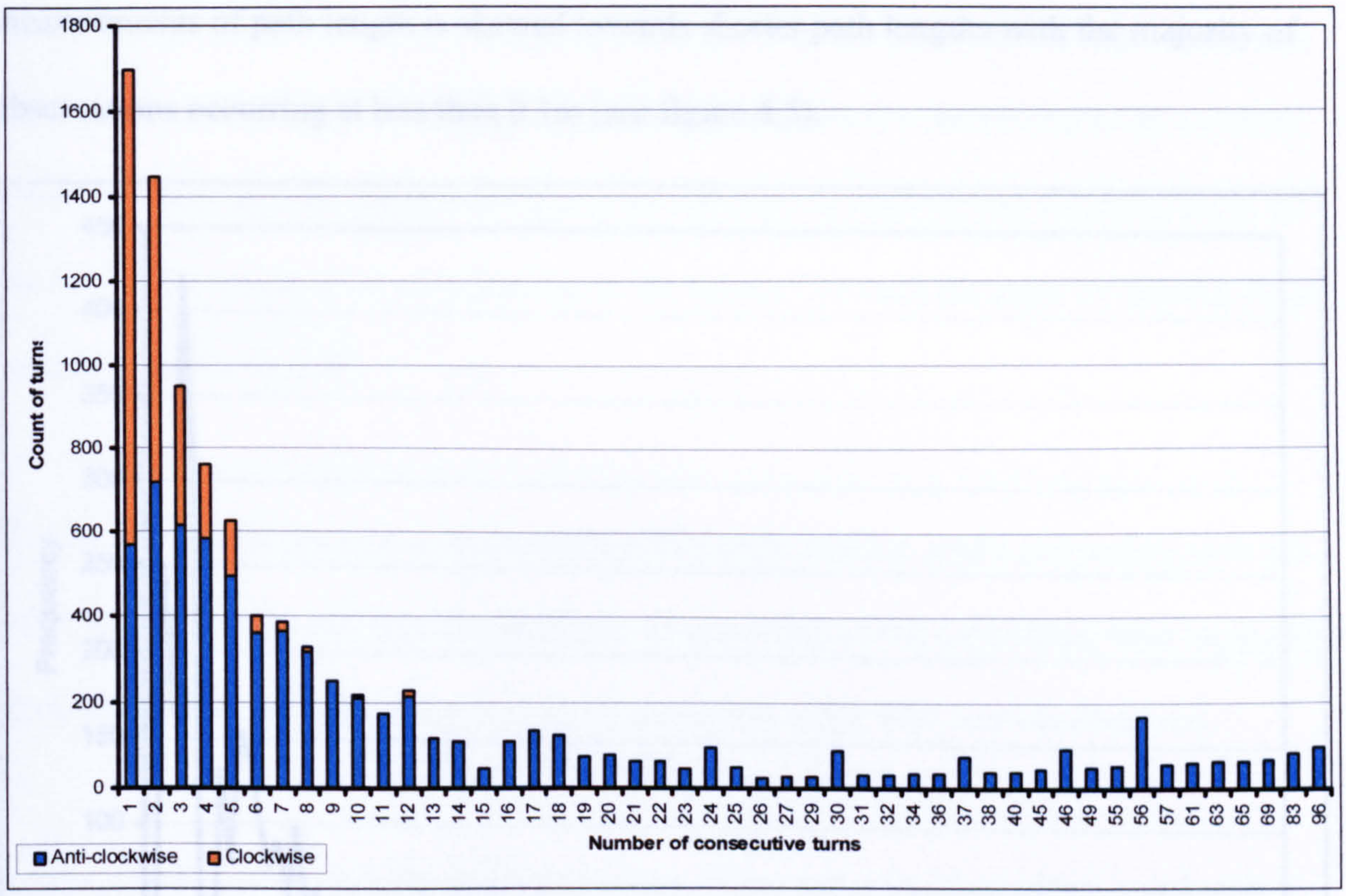

Figure 4.4 – The number of consecutive rotations in a clockwise or anti-clockwise direction.

The pattern of clockwise and anti-clockwise rotations and the number of rotation events recorded for each vine weevil over the two day period is highly variable (see figure 4.3). With a few exceptions, however, the data does suggest that the vine weevil is directionally biased towards anti-clockwise rotation ($t=8.06$, $P<0.001$ from paired t-test of anti-clockwise to clockwise rotations by individual vine weevil).

The bias towards anticlockwise rotation is also prevalent in the relative length of consecutive turns (see figure 4.4). Clockwise rotations are skewed towards single rotation events with all sequences less than 13 turns. Anti-clockwise sequences are considerably more common with instances of up to 96 consecutive anti-clockwise turns observed.

The cumulative frequency of locomotory path length is highly distorted by the number of interactions with the edge of the arena. Of 10,000 observations 7,158 resulted in an encounter with the edge of the arena. The cumulative frequency of the remaining

measurements of path length is skewed towards shorter path lengths with the majority of observations occurring at less than 0.1m (see figure 4.5).

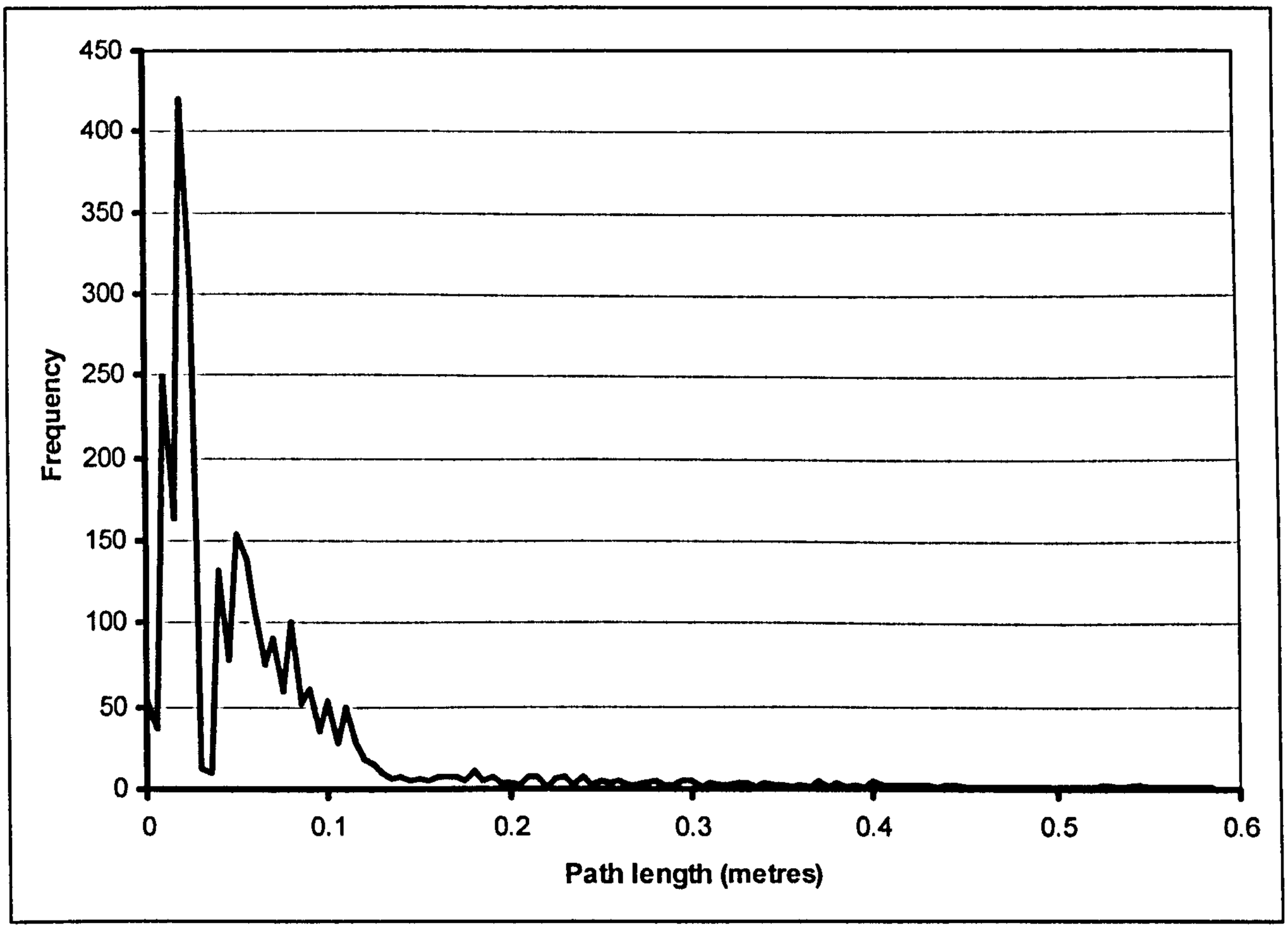

Figure 4.5 – Cumulative frequency distribution of path length of 45 vine weevils measured in a 30cm diameter arena.

4.3.3. Possible errors in the experimental data

There are four possible problems with the experiments that bring some doubt to the results obtained.

The first experiment was based upon the observation of six vine weevil adults. The vine weevils used in the experiment (the only adults available at the time) were collected at the same time and from the same place - as such they are likely to be siblings. They were also the batch upon which the initial observations of directional bias were made. The observations made, therefore, may not be representative of vine weevils in general.

Secondly, the method of measurement of the angle of rotation introduces some doubt into the accuracy of the results. Time pressure, the application of a flat protractor to a convex monitor screen, and the slight fuzziness in interpreting the starting and finishing angle of rotation add a certain level of variability to the results. As such the angle of rotation must be considered to be $\pm 10^{o}$.

Thirdly, the size of the arenas utilised was sufficiently small to make encounters with the arena walls a regular and significant effect. This artefact of the experiment may engender a significant effect upon the behaviour and locomotion of the vine weevils observed.

Finally, there is the possibility of observer bias. This (subconscious) effect could shift perception of, and subsequently recording of, an angle away from 90^{o}, 180^{o}, 45^{o}, etc. Alternatively it could produce "familiar number" recognition resulting in exacerbated spikes (with positive discrimination) or flattening (with negative discrimination). The frequency distribution shows a marked bias towards whole numbers. A paired t-test was performed to compare the 10's with the 5's, i.e. 10^{o} compared with 15^{o}, 20^{o} with 25^{o}, etc - this yielded a $t=4.80$, $P<0.001$. This problem, therefore, could add a further $\pm 10^{o}$ to the error in measurement. A similar effect in the measurement of path length may also distort the frequency distribution of path length.

Points two and three will only affect the angle measured and not the observation of the direction of rotation. Summation of the data into 90^{o} categories, grouping the observations beyond the observer bias sensitivity, diminishes the impact of the observer bias. Using this approach the relative distribution of angle of rotation is skewed towards angles of less than 90^{o}.

Random does not mean sequential. The probability of finding a sequence of n runs in x number of observations with a bias of b is governed by the equation:

$$P(sequence) = x.\left(\frac{1}{b}\right)^{-n}$$

Thus to achieve a sequence run of 96 consecutive anti-clockwise rotations in 10,000 observations requires a bias of approximately 81%. The mean bias for the second experiment was 71.8% with a standard deviation of 17.37%. The distribution of the length of sequential runs, therefore, could be random.

4.4. Discussion

Three possible benefits could be derived from directionally biased locomotion.

The first benefit is the simplification of the decision making process. If, in a given context, the decision of whether to rotate in one direction or another is pre-determined, the neural processing power required would be reduced. Consequently the speed of response and the resources employed would be reduced. Specific routines would only have to be developed for one sequence of application of limbs.

The second benefit relates to the possibility of the formation of spirals. Spirals will form under conditions of directional bias if the angle of rotation predominantly less than 90^{o} and if the angle of rotation or the path lengths between rotations tend to increase with every rotation. Provided the period of the spiral (the distance between successive passes of the same sector) is at the optimal detection distance, the spiral forms an optimal detection/foraging pattern.

The third benefit relates to the vine weevils almost prescient ability to exploit any gap or hole in a container to escape. Directional bias, with an angle of rotation predominantly below 90°, enables the systematic following of edges. Thus vine weevil seeking to find a way out of (or a way into) a given container will systematically follow the edges of the different planes of the container until a gap or hole is found.

Chapter 5. - Video observation of movement and behaviour of *Otiorhynchus sulcatus* in an arena virtually of infinite dimensions (AVOID).

5.1. Introduction

The observation of movement and behaviour in the previous chapter was influenced by the form and constraints of the use of a traditional arena. Measurement of path length was curtailed by collision with the walls of the arena and limited to observations less than a fraction of the diameter of the arena. If the angle of rotation and directional bias is a function of the escape and evasion behaviour of the vine weevil then these measurements may also be detrimentally influenced by the arena design.

In order to understand and measure the motion and behaviour of the vine weevil an arena without a definitive physical border is required. The most practical method of achieving this is to build an arena that is spherical – an arena that has a surface without any walls – an Arena Virtually Of Infinite Dimensions.

A series of three experiments were conducted to compare the unconstrained movement of vine weevil.

1. Comparing movement during the pre-oviposition period with movement after oviposition has occurred.

2. Comparing movement of vine weevil kept in large groups with vine weevil kept singly.

3. Comparing movement of unfed vine weevil with vine weevil fed leaves with either high N application or with a low N application to the host plant.

5.2. Methodology

5.2.1. Arena virtually of infinite dimensions (AVOID)

Two practical methods of creating an AVOID are possible. The first is to use a sphere with the test subject inside - a sphere made from some transparent material – within which the vine weevil can move in any direction. The second is to use a sphere with the test subject on the outside surface. For the purposes of this experiment the AVOID was of the second variety.

The AVOID consists of a 0.5m diameter sphere supported upon four ball bearings and rotated using two computer controlled motors at ninety degrees to one another (see figure 5.1). A light box and video recording system of similar design to the previous chapter was then built around the apparatus.

Observations of the movement of the weevils were recorded to the hard disk of a computer using a standard USB "web-cam" video camera. The weevils were kept centred within the view of the camera by rotating the AVOID sphere in the equal but opposite direction to movement of the vine weevil under investigation using X-axis and Y-axis signals sent to the electric motors.

Distance travelled by the vine weevil was then determined by correlating the starting and concluding points of a path with numbered reference points labelled in a dodecahedral pattern on the AVOID sphere. The angle of rotation was then determined by the difference in direction between sequential paths.

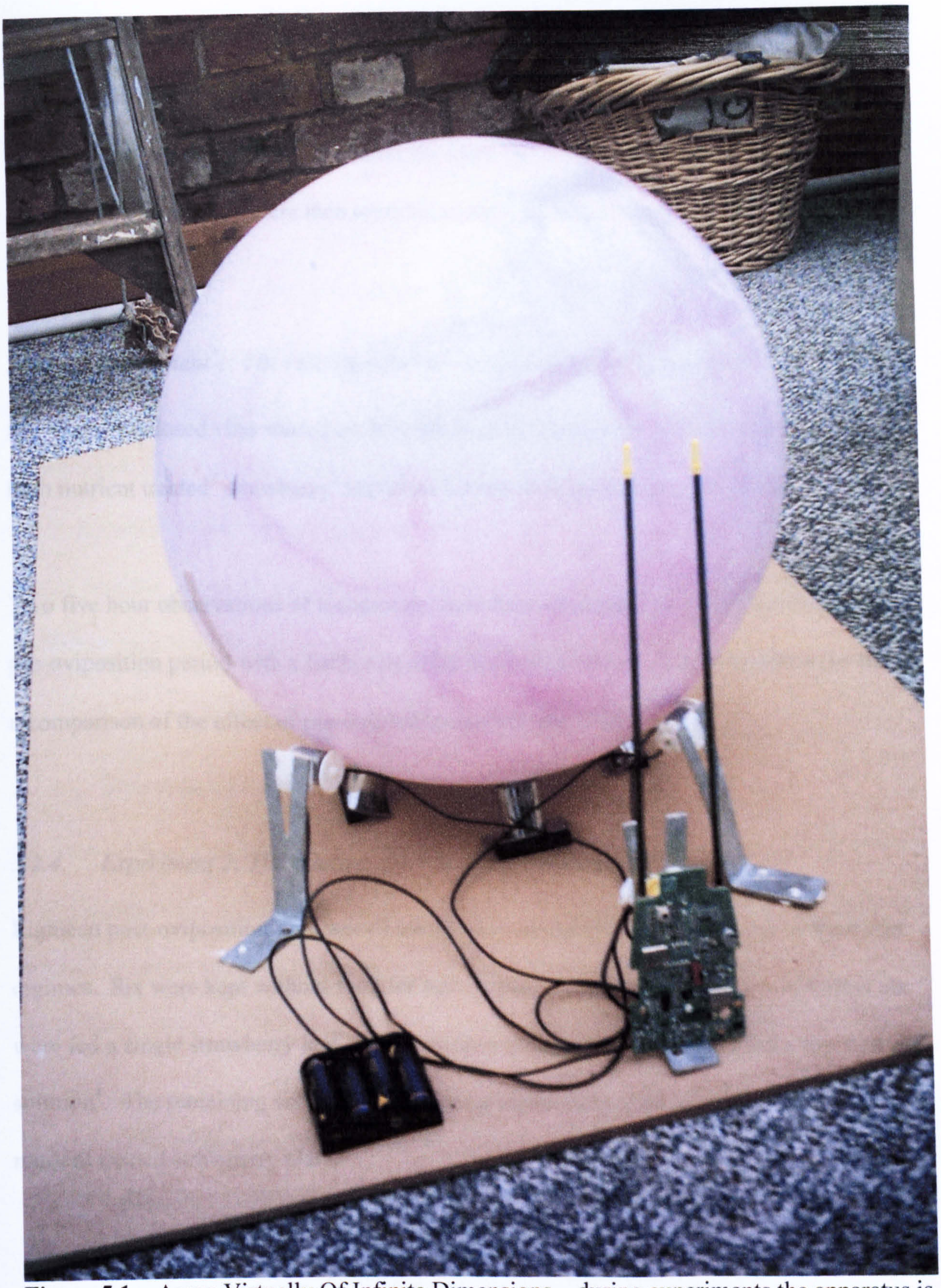

Figure 5.1 – Arena Virtually Of Infinite Dimensions – during experiments the apparatus is covered by a light box containing a faint light source and a digital camera.

5.2.2. *Observations and Light:Dark cycle*

All vine weevils were kept in a constant 16:8 day:night cycle throughout the experiment. Observations were made two hours after the night cycle began with observations lasting five hours. The weevils were then returned to their pre-observation regimen.

5.2.3. *Experiment 1: The relative effect of oviposition upon locomotion*

Six recently eclosed vine weevil adults were kept in isolation at room temperature and fed high nutrient treated[2] strawberry[3] leaves *ad libitum* throughout the experiment.

Two five hour observations of locomotion were then conducted for each weevil during the pre-oviposition period with a further two five hour observations after oviposition (to enable a comparison of the effect of pre-oviposition period upon locomotion).

5.2.4. *Experiment 2: The relative effect of diet upon locomotion*

Eighteen post-oviposition vine weevil adults were randomly allocated to one of three diet regimes. Six were kept without food for two weeks prior to an observation. A further six were fed a single strawberry leaf every five days from a strawberry plant fed a low nutrient solution[4]. The remaining six adults were fed *ad libitum* strawberry leaves from a high nutrient treated strawberry plant.

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[2] 100% manufacturers recommended concentration of miracle gro®

[3] CV Red Gauntlet

[4] 10% manufacturers recommended concentration of miracle gro®

Each weevil was then observed during two five hour observation runs. The unfed weevils were observed on consecutive days (to reduce mortality). All other observations of individual weevils were separated by a week.

5.2.5. Experiment 3: Relative effect of crowding upon locomotion

Twelve post-oviposition vine weevil adults labelled with a number super glued to their backs. The weevils were then randomly allocated to two treatment regimes. Six were kept in individual isolation with each weevil being transferred to a different sterile container every day prior to observation. The remaining six were kept together within the same 50mm diameter container for two weeks prior to observation. All weevil adults were fed *ad libitum* high nutrient supplied strawberry throughout the experiment.

5.2.6. Data collection and analysis

For each of the experiments observations of the pattern of locomotion were made and measurements were taken of; the relative directional bias, angle of rotation, path length, the duration of non-movement, the maximum distance from the origin and speed of locomotion.

The proportion of rotations that were anticlockwise (compared to clockwise rotations and excluding non-rotation pauses) was determined for each observation run for each of the experiments – this proportion was then arcsine transformed before further analysis.

Path length for each experimental run was determined by trigonometric calculation of location of starting a locomotory period and finishing a locomotory period, with reference to marked positions on the surface of the AVOID apparatus. The path length was then

determined from the difference between the two points (allowing for multiple orbits of the AVOID apparatus).

Angle of rotation was determined trigonometrically from the angle of difference between two consecutive paths as established by reference to the marked fixed positions on the AVOID apparatus.

The distance from origin was determined by calculation of the hypotenuse from the cumulative "X" and "Y" co-ordinates. For analysis the maximum distance from the origin was used.

The speed of locomotion, as determined by the distance travelled divided by the duration of locomotion, was compared for each of the treatments for the three experiments.

All experiment one observations were analysed using a paired t-test – matching individual vine weevils pre-oviposition and post oviposition. All experiment two observations were analysed using a one-way ANOVA with Tukey's as a post test if a significant difference was found. All experiment three observations were analysed using a two sample t-test.

5.3. Results

5.3.1. Description of pattern of locomotion

The path made during each of the observations lack a consistent or coherent structure. Some form vaguely globular or linear formations, or combinations of both. No particular structure or formation could be related to, or attributed to, a particular pre-experimental treatment. Spirals were not evident. Structure is strongly influenced by both angle of rotation and path length. Please see appendix 2 for path plots.

5.3.2. Directional bias

The paired t-test comparing pre-oviposition period locomotion of vine weevil with post-oviposition locomotion of the same weevil yielded a value of t= -1.56, P=0.179, suggesting that the proportion of directional bias is not significantly different between the two life history periods.

The three feeding regimes were also found to not materially affect the proportion of directional bias (one way ANOVA, F=1.18, df=2, P=0.335).

Grouping of vine weevil prior to observation was also found not to significantly affect the proportion of anticlockwise rotations (two sample t-test t=-0.30, df=8, P=0.773).

5.3.3. Angle of rotation

It was found that the pre-oviposition and post-oviposition weevil could not be differentiated with respect to angle of rotation (T=-0.86, P=0.428).

The second experiment, comparing levels of feeding prior to observation, also failed to reveal a measurable difference in angle of rotation for the three treatments (one way ANOVA, F=0.72, df=2, P=0.495).

Angle of rotation for adult weevil kept isolated prior to observation were also found not to be significantly different from weevil kept in groups (two sample t-test T=0.29, df=13, P=0.773)

5.3.4. Path length

The path length of each step was found to be unaffected by each of the treatments. A two sample t-test of pre-oviposition to post-oviposition gave T=-1.65, P=0.159. One way ANOVA of feeding regime gave F=2.23, df=2, P=0.123. Two sample t-test comparing grouped and un-grouped weevil gave T=0.90, df=13, P=0.383 with respect to path length.

5.3.5. Duration of non-movement

Post-oviposition vine weevil had a mean period of non-motion between movement of 58 seconds, which was significantly greater than the 18 seconds of pre-oviposition vine weevil (two sample t-test, t=3.10, P=0.027).

Frequency analysis of duration of non-movement showed that the difference between the two treatments is predominantly a consequence of a difference in frequency of events of

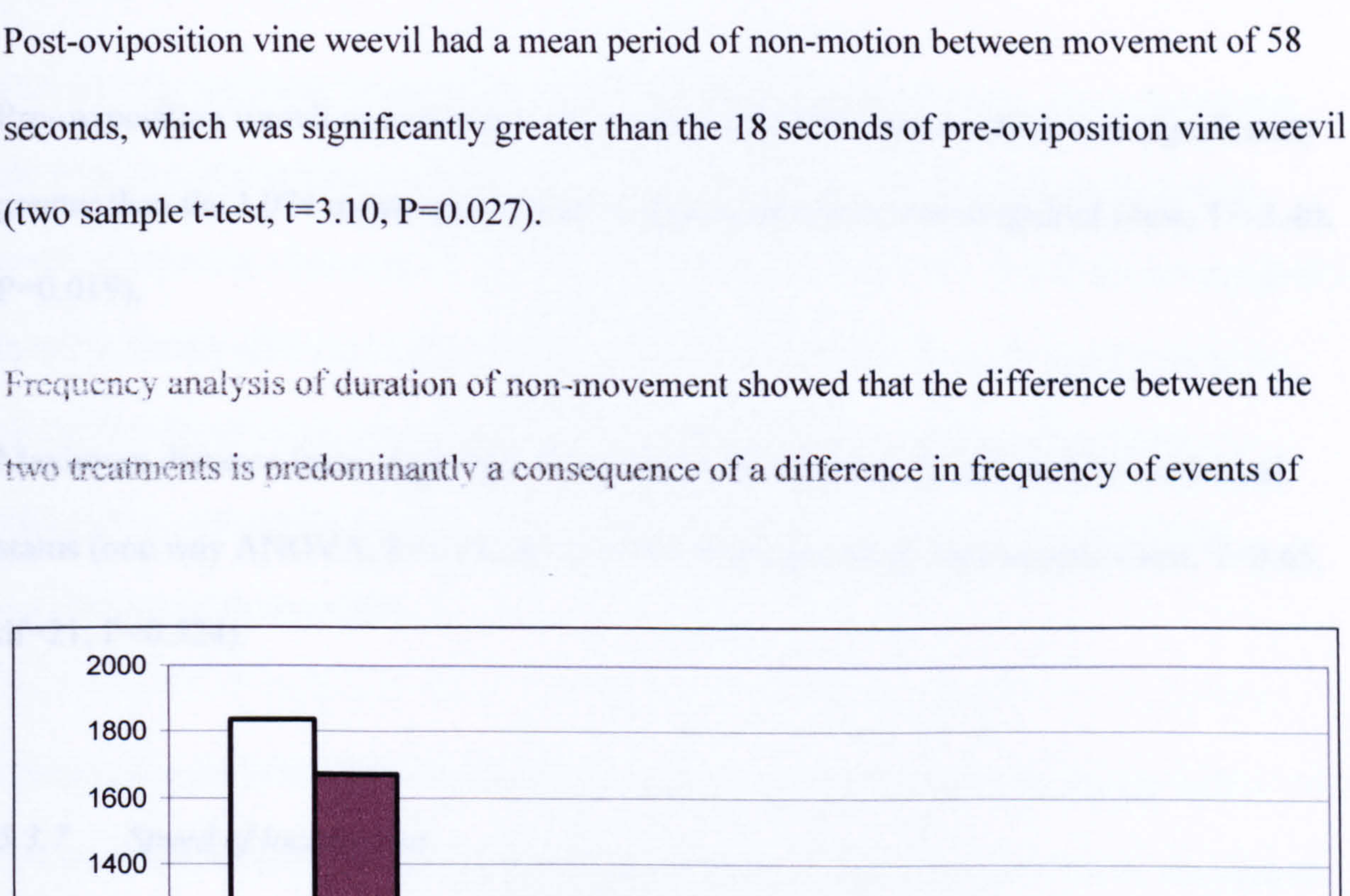

Figure 5.2 – Frequency distribution of duration of periods of non-movement for observations taken Pre-oviposition and Post-Oviposition.

Nutritional status was not found to have a significant effect upon duration of non-movement (one-way ANOVA, F=1.72, df=2, P=0.213). Duration of non-movement was also found not to be affected by grouping (two sample t-test, T=0.98, df=5, P=0.372)

5.3.6. Maximum distance from origin

The maximum distance that each vine weevil had travelled away from the origin was determined for each trial.

Pre-oviposition weevil were found to have travelled 2.097 metres which was significantly greater than the 1.074 metres performed by post-oviposition weevil (paired t-test, T=-3.40, P=0.019).

Maximum distance from origin was found not to be significantly affected by nutritional status (one way ANOVA, F=1.13, df=2, P=0.334) or grouping (two sample t-test, T=0.65, df=21, P=0.524).

5.3.7. Speed of locomotion

Two sample t-tests for pre-oviposition vs. post-oviposition and the effect of grouping failed to reveal a significant difference between the two groups (T=-0.59, P=0.578 and T=0.18, df=16, P=0.863 respectively).

Vine weevil adults fed a low nutrient treated leaf were found to be significantly slower than high nutrient treated leaf fed, although starved weevil was found to be not significantly different from the other treatments (one way ANOVA, F=5.53, df=2, P=0.008 – difference revealed by Tukey's pairwise comparison).

5.4. Discussion

5.4.1. Comparison of AVOID apparatus to traditional arena

The AVOID apparatus used in these experiments clearly demonstrate the inadequacies of a traditional arena in the study of locomotion. Path lengths have been measured that are two orders of magnitude greater than those possible with a traditional arena. The frequency of turning and directional bias has also been shown to be slightly less than experienced with traditional arenas.

The AVOID apparatus is not without some idiosyncrasies and foibles. It was found that the motion of the ball can be jerky. The jerks, on some occasions, seemed to have the result of inducing a locomotory event in the vine weevil observed. Induced motion appears to occur shortly after the vine weevil has stopped moving and the ball is still rotating to centre the weevil relative to the camera. When the ball then stops the weevil has a tendency to start walking again. Practice and anticipation of movement reduced the occurrences of these events.

Certain changes in direction are difficult to emulate with the motors driving the ball. Certain amplitudes of zigzags, allowing the weevil to almost leave the view of the camera in one axis, before compensating with the other axis control reduces ball shudder. Long periods of smooth movement without stopping are the key. The apparatus could also be improved by significantly increasing the diameter and mass of the ball.

The AVOID apparatus lends itself readily to automation. Visual recognition systems and reactive robotic toys are now commonplace. If these can be appropriated into an automated AVOID apparatus it may be possible to collect behavioural data at statistically viable scales.

5.4.2. Locomotion and oviposition – Pre-oviposition vs. Post-oviposition

Period of time without motion and maximum distance from origin were found to be statistically different between the two treatments. It is difficult to understand how an average time differential of 7 seconds could benefit the vine weevil.

It is expected that this result is an artefact of the experiment. Pre-oviposition observations were amongst the first observations made. Post-oviposition observations were amongst the last. The level of skill involved in operating the AVOID apparatus increased during period of the experiments (as previously discussed). It is expected, therefore, that an increased level of juddering resulted in a decrease in pre-oviposition non-movement duration.

Further investigation – possibly with automated AVOID apparatus – will have to be conducted to verify (or more likely disprove) the disparity in duration of periods without movement.

Increasing the rate of dispersion by 50-60% (as evidenced by the disparity in maximum distance from origin) would be of great importance to the vine weevil. Increased dispersal would reduce competition with siblings. Reduced dispersal when ovipositing would improve the probability of ovipositing in the same area as feeding.

Unfortunately this observation, though practical to the vine weevil, could also be an artefact of the increase in proficiency in the operation of the AVOID apparatus. Post-oviposition vine weevil spend 18% less time actually moving – possibly due to the operation of the AVOID. It would be expected, therefore, that there would be a concomitant increase in maximum distance from origin. This observation is further confounded by the observation that disturbance tends to result in locomotion in the same direction. Reduced incidence of turning will tend to increase the distance travelled.

5.4.3. Locomotion and nutritional state

The only measurement found to vary between the treatments was speed of locomotion. The practical benefit to the vine weevil and the possible mechanism for this occurrence are unknown. Particularly perplexing is why an intermediary diet would have a more profound effect upon speed than a higher quality or absence of food altogether.

5.4.4. Locomotion and isolation

Vine weevil adults appear to be indifferent to grouping or isolation with regard to locomotion. The effect of grouping, however, could be labile with regard to time. If any variances in locomotion are inherent within the vine weevil due to crowding – removing the weevil for observation may quickly or instantaneously reset the behaviour.

Unfortunately the AVOID apparatus is not a practical tool for measuring locomotion of more than one individual at a time.

Chapter 6. - Determination of locomotive capabilities of larval stage *Otiorhynchus sulcatus*

6.1. Introduction

Hibbard (2004) found that the larvae of *Diabrotica virgifera virgifera* LeConte were capable of significant movement down and across plant rows (0.76 metres between rows) suggesting that larval locomotion is undervalued as a mode of dispersal and also that patch sizes may be larger than anticipated for root feeding insect species.

The previous experiments have assumed that the definitive unit of reference for an oviposition site is that of a single host plant contained within a plant pot. Given that the plant pot is a relatively recent introduction - assumed to be a period of time not evolutionarily significant to *Otiorhynchus sulcatus* – it is possible that the unit of reference for an oviposition site could be greater than a single plant. Host plants, under natural conditions, would be expected to form a subterranean network of intermingled roots. It would not be expected that each plant would have a discrete root zone physically isolated from its neighbours. It is possible that the patch (expected to be inhabited by the larvae resulting from a clutch oviposited at a particular site) could be greater than a single plant. Optimal oviposition, therefore, could be greater than the potential carrying capacity of the host plant even when allowing for mortality and developmental failures.

Even if the host plant root systems are discretely spaced the creation of a physical barrier preventing movement from one host to another is a recent introduction. Larval locomotion and dispersal, therefore, would be an important determinant of patch size. Larval locomotion would thus be an important evolutionary factor in clutch size determination and oviposition behaviour.

The purpose of the following experiments is to determine the locomotory capability of the larval *O. sulcatus* and relate these observations to patch size, clutch size and oviposition.

6.2. Methodology

In each of the experiments; sixteen Perspex cubes with internal dimensions of 30cm, open on one face, were half filled with peat based potting compost. A third instar vine weevil larva was then placed in the centre of each cube and the cube filled with compost.

After a specific period (six hourly for the first experiment and hourly for the second) the perimeter of the cube is checked for any sign of the larva. If the larva is found at the perimeter, the position is noted, the larvae excavated and replaced at the centre of the cube. In all other cases the cube is excavated in 1cm layers until the larva is found. When the larva is found, its coordinates are determined, the larva returned to the centre of the cube and the compost returned.

6.3. Results

All data was recorded in three dimensional Cartesian coordinates. The coordinates were then converted into distance from origin using Pythagoras' theorem. This was then converted into daily dispersal speed by dividing by the time period and multiplying by 24.

6.3.1. Experiment 1: Observations every 6 hours over seven days

Mean daily dispersal speed of the larvae was found to be 0.496 metres per day with a standard deviation of 0.173m.day^{-1}.

At this speed a third instar vine weevil larva would be expected to traverse approximately 125mm in the six hour period between observations. 55 of the 464 observations, however, revealed a larva at the edge of the cube. This indicates that the apparatus, in the time period utilised, constrains the larva, reducing the distance travelled and thus the dispersal speed measured is underestimated. To combat this, a further experiment was conducted, with observations every hour over a twenty four hour period.

6.3.2. Experiment 2: Hourly observations over 24 hours

Mean daily dispersal speed of the larvae was found to be 0.854 metres per day with a standard deviation of 0.301m.day^{-1}.

No larvae were found at the edge of the apparatus during the experiment which suggests that the time frame was sufficiently short to prevent encounters with the edge.

6.4. Discussion

The experiment demonstrates the considerable mobility of vine weevil larvae in un-compacted peat based compost. It is expected, however, that mobility through denser, more compacted media, would be greatly reduced.

Although the dispersal speed of vine weevil larvae is considerably less than that of the adult, the dispersal speed is great enough to consider the size of the patch to be greater than that of a single plant. Assuming that the rate of dispersal is similar for feeding and non-feeding larvae then, given an unbroken interlinking root system, a speed of 0.8m.day^{-1} and a development time of 200 days this would yield a potential patch size of approximately 320 metres in diameter.

If the root systems were discrete then the size of interstice that could be spanned would be related to the survival time (excluding disease and predation) of the larva. Thus maximum traversing distance would be a function of the metabolic reserves held by the larva divided by the daily metabolic requirements. Personal observations of third instar larvae showed that larvae deprived of food for two weeks resumed feeding and development. Assuming that larvae are capable of movement throughout this period – it is possible for a larva to traverse interstitial distances of more than ten metres.

Based upon these observations it is believed that oviposition behaviour and clutch size evolution are unlikely to be constrained by the need to complete the entire larval life cycle upon a single plant. It is expected, therefore, that oviposition at a given host plant will be greater than the potential carrying capacity of that plant.

An unfortunate side effect of this strategy (both for the vine weevil and for the horticultural industry) is the introduction and utilisation of containers and plant pots. Within a pot the mobility of the larva is artificially constrained - preventing dispersal when the root systems are damaged beyond the economic (to the vine weevil) threshold. It is not in the best interests of the vine weevil to kill the host plant before reaching maturity. The absolute threshold, therefore, would be reached when feeding kills the plant before any of the larvae pupate.

The potential disaster to the vine weevil resulting from containerisation is alleviated partially by other horticultural practises. Sub-optimal insecticide usage, resulting in decimation rather than extermination, may result in adjusting the population below the threshold, allowing some of the larvae to reach adulthood. Additionally; the use of fertilisers increases the quality of the host root system. This enables the plant to survive longer - generating more and/or higher quality roots to feed vine weevil.

Chapter 7. - The use of molecular genetics of *Otiorhynchus sulcatus* for the determination of oviposition site sharing.

7.1. Introduction.

Is there an underlying mechanism that prevents or discourages vine weevil adults from ovipositing at the same site? Site discrimination, as a method of reducing competition between clonally related individuals would be an effective evolutionary stable strategy, supportive of Hamilton's Rule and consequently of considerable importance to the understanding of dispersal and population expansion of *Otiorhynchus sulcatus*. The discovery of site discrimination in vine weevil would be indicative of either highly efficient dispersion and/or suggestive of the presence of a marker at the host site "warning" future weevil against using the site. The possibility of a secreted marker, from either the ovipositing adult/ova or from the host plant, would be of considerable importance as a potential control agent.

In order to determine whether the ova at an oviposition site are the product of single or multiple adults a method for differentiating between the ova of different adults is needed. Two methods are available for this type of investigation: labelling adults with stable/radioactive isotopes, or, the use of nucleic acid differentiation techniques.

7.1.1. Isotopic labelling

Isotopic labelling is degenerative in nature. The signal difference between wild and labelled individuals diminishes with time due to natural biological cycles. Isotopic labelling is highly dependent upon the retention, and later sequestration, of bodily reserves to the newly formed ova, of the isotope used. If the level of sequestration were very low, with nearly all of the elements that make up the newly formed ova coming from recently

ingested material, the signal would be undetectably low. In order for this methodology to be effective, an extremely large dose of the label would have to be administered to the adult prior to ovigenesis. Fecundity is highly correlated with N intake (Cram, 1965; Maier, 1981). It is expected, therefore, that sequestration is low, with ova created predominantly from recently ingested material. For this reason isotopic labelling was eliminated as a potential methodology.

7.1.2. Differentiation using nucleic acid variation

The two methods of genetic labelling and the investigation and exploitation of extant genetic differences were considered. Given the political and technical difficulties in introducing a genetic label to a eukaryotic organism, the discovery and exploitation of extant genetic differences was determined to be the most feasible tool for the investigation.

0. sulcatus is thought by many to reproduce exclusively by parthenogenesis (Moorhouse, 1992; Smith, 1932). As a parthenogenic organism genetic variation in a population will only occur through the process of mutation. Consequently, any study of molecular genetics of *0. sulcatus* must be one of mutation analysis. Standard Mendelian genetics and numerical analysis are of limited value.

When considering a method of genetic analysis three factors have to be taken into account: the sensitivity of analytical technique, rate of mutation, and the characteristics of the target organism's DNA.

7.1.3. Molecular biology of the mitochondrion.

The mitochondrion is the sub-cellular organelle responsible for the biochemical function of respiration. Each eukaryotic cell contains large numbers of individual mitochondria. The

actual number of mitochondria is dependant upon the species of the organism, the function of the cell and the biochemical state of the cell (Alberts *et al.* 1989).

It is thought by many that the mitochondrion has evolved from the symbiotic inclusion of prokaryotic organisms (similar to the modern azotobacter and cyanobacter) into eukaryotic host cells (Alberts *et al.* 1989; Hawkins, 1996). As a result of their independent past mitochondria have a number of unusual traits.

Mitochondria contain multiple copies of circular chromosomes that code for all the RNA species and some of the proteins involved in the function of the organelle. This DNA is referred to as mitochondrial DNA (mtDNA). The codon usage within the mitochondrion also differs from that of nuclear DNA (Alberts *et al.* 1989; Hawkins 1996; Moritz *et al.* 1987; Primrose 1995). The rate of genetic mutation is significantly higher in mtDNA than nuclear DNA (Moritz *et al.* 1987). This is due in part to the absence of proofreading enzymes and to the direct mutagenic action of "high-energy" electrons and free radicals associated with respiration in the mitochondrion (Alberts *et al.* 1989).

Mitochondria divide and replicate partially independently of their cellular host. The inheritance of mitochondria is cytoplasmic and non-Mendelian. The number, and probability of inclusion of individual mitochondria (and their mtDNA), is related to the amount of cytoplasm donated by the parent cell and the proportion of different mitochondria present in the donated cytoplasm (Rand and Harrison 1986). For example, in mammals, sperm contributes very little cytoplasm to the newly fertilised ova, and thus inheritance of mitochondria and mtDNA is almost exclusively maternal. In other species where some paternal contribution is observed, the paternal contribution to mitochondrial inheritance is referred to as "paternal leakage". During cell division the two daughter cells receive approximately fifty per cent of the parent cell's cytoplasm (Rand and Harrison

1986). The number of mitochondria and the type of mitochondria are thus likely to be similar in daughter cells and parent cells (Rand and Harrison 1986).

Two distinct mechanisms, the loss of mitochondrial genomes by genetic drift and the formation of new mitochondrial genomes by mutation, maintain heteroplasmy in several species studied (Harrison *et al.* 1985; Moritz *et al.* 1987; Rand and Harrison 1986; Solignac *et al.* 1984; Solignac *et al.* 1987; Solignac *et al.* 1986). The process of genetic drift would therefore eventually result in homoplasmy; however, mutation is constantly producing "new" mitochondrial genomes, resulting in a heteroplasmic stalemate. Thus mutation, and the pattern and function of inheritance, result in varying degrees of mitochondrial heteroplasmy in all species and individuals (Hawkins 1996; Moritz *et al.* 1987).

7.2. Extraction of DNA from ova - Can sufficient DNA be extracted from an ovum for investigation and differentiation of mtDNA?

7.2.1. Method

DNA was extracted from six vine weevil ova using a standard phenol-chloroform extraction with cold ethanol precipitation (Sambrook *et al*, 1989). The precipitated DNA pellets were then re-suspended in 100µl of TE (Tris-EDTA). DNA yield was then quantified using UV absorbency at 260nm and by the ethidium bromide comparison of prepared standards (Sambrook *et al*, 1989).

7.2.2. Results

	UV absorption at 260nm	Ethidium Bromide comparison	DNA/Ova
1	4.25 ng/µl	<5 ng/µl	425 ng/ova
2	10.75 ng/µl	>10 ng/µl	1075 ng/ova
3	10.20 ng/µl	<10 ng/µl	1020 ng/ova
4	8.25 ng/µl	<10 ng/µl	825 ng/ova
5	3.30 ng/µl	<5 ng/µl	330 ng/ova
6	6.00 ng/µl	>5 ng/µl	600 ng/ova
Mean	**7.13 ng/µl (±3.10)**		**713 ng/ova (±310)**

Table 7.1 – Quantification of DNA phenol-chloroform extracted from vine weevil ova by UV absorption at 260nm and ethidium bromide comparison.

7.2.3. Discussion

The two methods of quantification correlate within the accuracy of their measurement. Standard PCR requires 25ng of DNA. The phenol-chloroform method, therefore, provides sufficient DNA for at least 10 investigations involving amplification with the possibility of further refinement improving yields.

7.3. Investigation of vine weevil mtDNA

7.3.1. Method

Standard PCR amplification (Anon, 1995) using combinations of primers specifically targeted to invertebrate mtDNA (Fleming, 1995) were used to amplify vine weevil mtDNA. DNA from the nematode *Globobdera palida* was used as a positive control. The PCR products were separated on a 1.2% agarose gel and the size of the bands compared with ϕX174 HaeIII digested DNA standard. PCR was performed on both DNA extracted

from individuals and from cocktails of DNA from multiple individuals to determine the existence size polymorphisms.

7.3.2. Results

The results are presented in the form of a table comparing: the predicted product expected from *Drosophila yakuba* (Mazars *et al*, 1991), the position on the *D. yakuba* mtDNA map and the primer designation used. The genes associated with the fragment in *D. yakuba* are also listed (Mazars *et al*, 1991).

The size of the amplified fragment is estimated to the nearest 50 base pairs. A designation of size designates that a product of that size has been produced at least once under optimised laboratory conditions. A designation of "no product" indicates that, despite optimised conditions, no product has been observed.

Table 7.2 – Amplification products from vine weevil mtDNA.

Primer	Pair	D yakuba position	Size (BP)	Gene	Product size
C1F3	C2R1	2798-3379	582	COI-COII	~600
	C02B	2798-3382	585	COI-COII	~600
	215R	2798-3685	887	COI-COII	~900
CO2A	C2R1	3124-3379	256	COII	~250
	CO2B	3124-3382	259	COII	~250
	215R	3124-3685	562	COII	~550
105CD	215R	3408-3685	278	COII	~300
	T6R1	3408-4125	718	COII-ATP6	~700
C2F3	T6R1	3685-4125	441	COII-ATP6	~450
C3F1	C3R1	4818-5304	486	COIII	~500
	C3R2	4818-5446	629	COIII	~650
C3F2	N3R1	5409-5724	316	COIII-ND3	~300
C3F3	N3R1	5648-5724	257	COIII-ND3	~250
	N3R2	5468-5919	452	COIII-ND3	No Product
N3F1	N3R2	5748-5919	172	ND3	No Product
	13B	5748-6384	637	ND3-tRNA phe	No Product
13A	13B	5946-6384	439	ND3-tRNA phe	~450
	N5R1	5946-6547	602	ND3-ND5	No Product
N5R2	N5F3	6888-7306	419	ND5	No Product
N4R2	N4F3	8501-8717	217	ND4	No Product
4LR2	N6R4	9652-10163	512	ND4-ND6	No Product
6	7	11564-11840	295	Cytb-ND1	No Product
ND1B	5	12249-12849	600	ND1-LrRNA	~600
	16S2	12249-12944	695	ND1-LrRNA	~700
ND1	5	12314-12849	535	ND1-LrRNA	~550
	16S2	12314-12944	630	ND1-LrRNA	~650
4	5	12586-12849	263	ND1-LrRNA	~250
	16S2	12586-12944	358	ND1-LrRNA	~350
LRR4	SRF3	13906-14361	456	LrRNA-SrRNA	No Product
AT2	AT1	14767-0030	1267	SrRNA-AT rich	No Product

7.3.3. Discussion

All products found in the investigation were approximately the same size as the equivalent *D. yakuba* mtDNA. It is expected therefore that the mtDNA in these regions is highly conserved, with respect to size, and thus not a viable candidate for exploitation as a differentiation tool.

The only regions that failed to yield PCR product were found in the ND regions and the AT-rich region. Thus one would expect there to be a difference between the mtDNA of *O. sulcatus* and *D. yakuba* within: 5724-5946bp, 6384-11840bp and 13906-0030bp. This may be as a result of HVR's (hyper-variable regions), poor suitability of the sequence for PCR (PCR works poorly on extended regions of AT sequences (Anon, 1995)), or, in the case of the expected 1267bp product, the sequence may be too long to amplify effectively with standard PCR.

All successful amplifications were characterised by a single size fraction. Even when the DNA of many weevils from very disparate locations was combined size polymorphism was not detected. Standard PCR of active mtDNA regions, therefore, cannot be used as an effective tool for the differentiation of vine weevil ova.

7.4. Insect mtDNA and the AT-rich region.

Insect mitochondria contain a non-coding region. This region forms the replication origin for the mitochondrial chromosome. This region is characterised by a high content of the bases adenine and thymine and is thus referred to as the AT-rich region. The high incidence of AT base pairs is very similar to the "TATA box" that forms the initiation site for many nuclear genes (Alberts *et al.* 1989).

Since the AT-rich region is non-coding, a significant amount of mutation can occur without affecting the function of the RNA and proteins coded in the mtDNA (Rand *et al.* 1994). Thus mutation, within the AT-rich region, is less likely to affect the function of the mitochondrion than the mutation of RNA or protein coding regions. Evolutionary selection pressure is more likely to act upon mutations that directly affect function (Rand *et al.* 1994). Consequently, mitochondria with AT region mutations are less likely to be affected by evolutionary selection than mitochondria with RNA and protein coding region mutation. Thus the probability of finding AT region mutations in daughter generations is greater than coding region mutations.

The AT-rich region is relatively simple in its sequence composition and thus prone to the formation of palindromic sequences. Palindromic sequences tend to produce changes in DNA strand length, either by inclusion of new DNA sequence, or by loss of sequence, between palindromes (Moritz *et al.* 1987; Rand and Harrison 1986). Simple sequence regions of DNA are also prone to replication slippage resulting in further changes in DNA strand length (Moritz *et al.* 1987).

It would be expected, therefore, that the insect AT-rich region would be highly size polymorphic. The literature supports this observation. The AT-rich region can vary in size by more than 20kb in an individual organism (Boyce *et al.* 1989). Despite the kinetic constraints of replicating larger DNA molecules, polymorphisms of DNA molecule size within an organism are retained and inherited by offspring. For example; of ten offspring of a heteroplasmic female cricket, all were heteroplasmic and appeared to have proportions of the two size variants similar to that of their mother (Harrison *et al.* 1985). However, in a later study (Rand and Harrison 1986) it was shown that shifts in genotype frequencies in the transmission from mother to offspring occur that suggest a bias in favour of smaller genomes.

7.5. The suitability of AT-rich region of mtDNA for 0. sulcatus genetic differentiation.

The AT-rich region of insect mitochondrial DNA is the most suitable for molecular genetic analysis of *0. sulcatus* for a number of reasons. In summary:

- Mutation rate is higher in mtDNA than in nuclear DNA.

- The mtDNA sequence is present in high copy number; since there are large numbers of mitochondria in each cell, each containing multiple copies of mtDNA.

- Independent reproduction of mitochondria results in a higher probability of mutation through inaccurate DNA replication in the mitochondria than in the nuclear genome.

- The AT-rich region of insect mtDNA is more prone to replication error mutation than coding mtDNA and nuclear DNA.

- Mutations in the AT-rich region of insect mtDNA are less likely to be affected by functional constraints and removed by evolutionary selection pressures.

- The multiple generations of mitochondria produced during cell division and replication result in a diversification of siblings' mitochondrial genetics during development to reproducing adults.

7.6. Sub-cellular ecology of mtDNA.

A theoretical model of the sub-cellular ecology was developed to predict the effectiveness of the AT-region size polymorphism as a molecular genetic technique. The logical analysis can then be used to estimate the outcome of an experimental approach and help refine experimental protocols.

The aim of the model and analysis is to deduce any theoretical differences (and predict the form in which the differences will take) between very closely related (potentially clonally related) individual organisms. If we begin the model with the assumptions that:

The number of cell divisions that separate two ova from a common progenitor cell is significantly less than the number of cell divisions that separate two adults (pre-ovigenesis). i.e. n(adult development)>>n(ovigenesis).

Mutation will cause a significant change in mtDNA composition which will be apparent over n_a cell divisions.

The rate of mutation will not be sufficient to significantly change the mtDNA composition after n_o cell divisions.

There are a finite, detectable number of mutant forms that the DNA can develop.

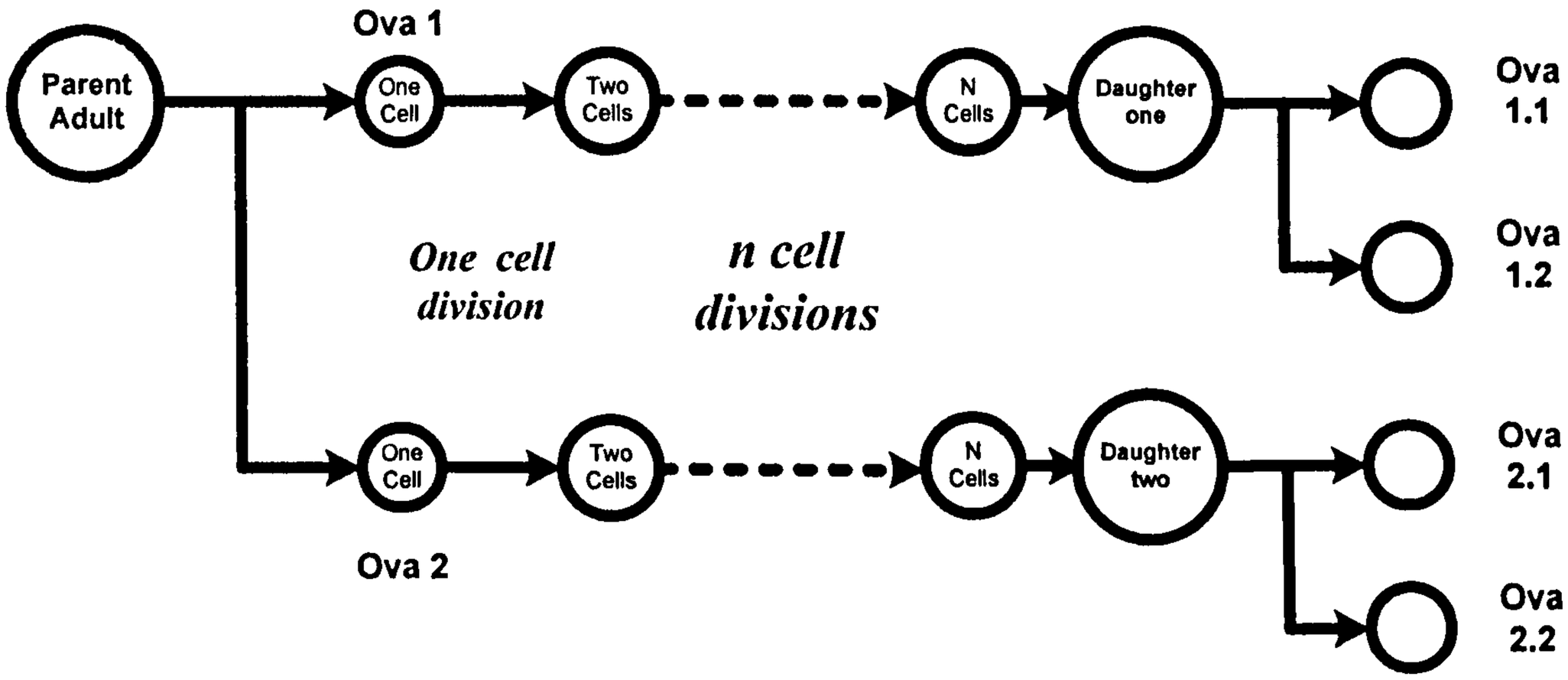

Figure 7.1 – Inheritance of mtDNA over a generation of cell divisions.

7.7. Model of mitochondrial inheritance in vine weevil

7.7.1. Preliminary equations

Where:

H = number of DNA species (polymorphism or heteroplasmic index).

x = total number of DNA molecules

Then heteroplasmic saturation will occur when the number of different DNA molecules equals the quantity of DNA molecules:

$$\left(\frac{H}{x}\right)_{\max} = 1 \qquad \textit{Equation 1.1}$$

Since the number of DNA species cannot exceed the number of DNA molecules, and there must be at least one DNA species, then:

$$1 \geq \frac{H}{x} \geq \frac{1}{x} \qquad \textit{Equation 1.2}$$

If we then introduce the terms:

h = rate of increase in number of DNA species

m = rate of production of new DNA species

d = rate of loss of DNA species

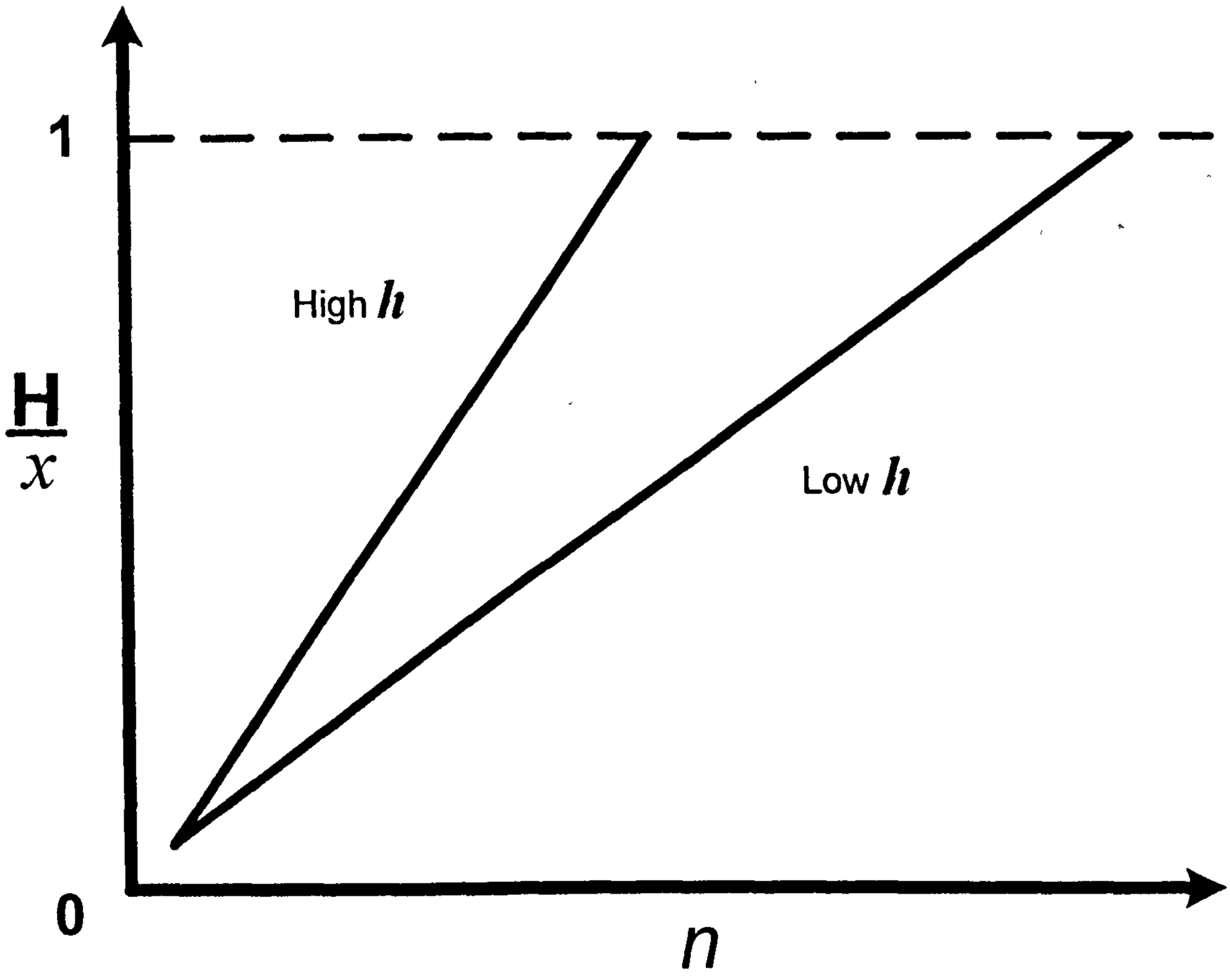

Figure 7.2 – Effect of *h* on time to reach heteroplasmic saturation

We can state that:

$$h \propto \frac{m}{d} \qquad \textit{Equation 1.3}$$

If we then introduce the term

n = Number of replications

Then, assuming that:

$$h \propto n \qquad \textit{Equation 1.4}$$

$$\frac{H}{x.n} \propto h$$

We can state that: *Equation 1.5*

$$\Rightarrow \frac{H}{x.n} \propto \frac{m}{d}$$

From *Equation 1.1*, heteroplasmic saturation, it can be deduced that as m increases (or d decreases) the number of replications required to reach heteroplasmic saturation decrease.

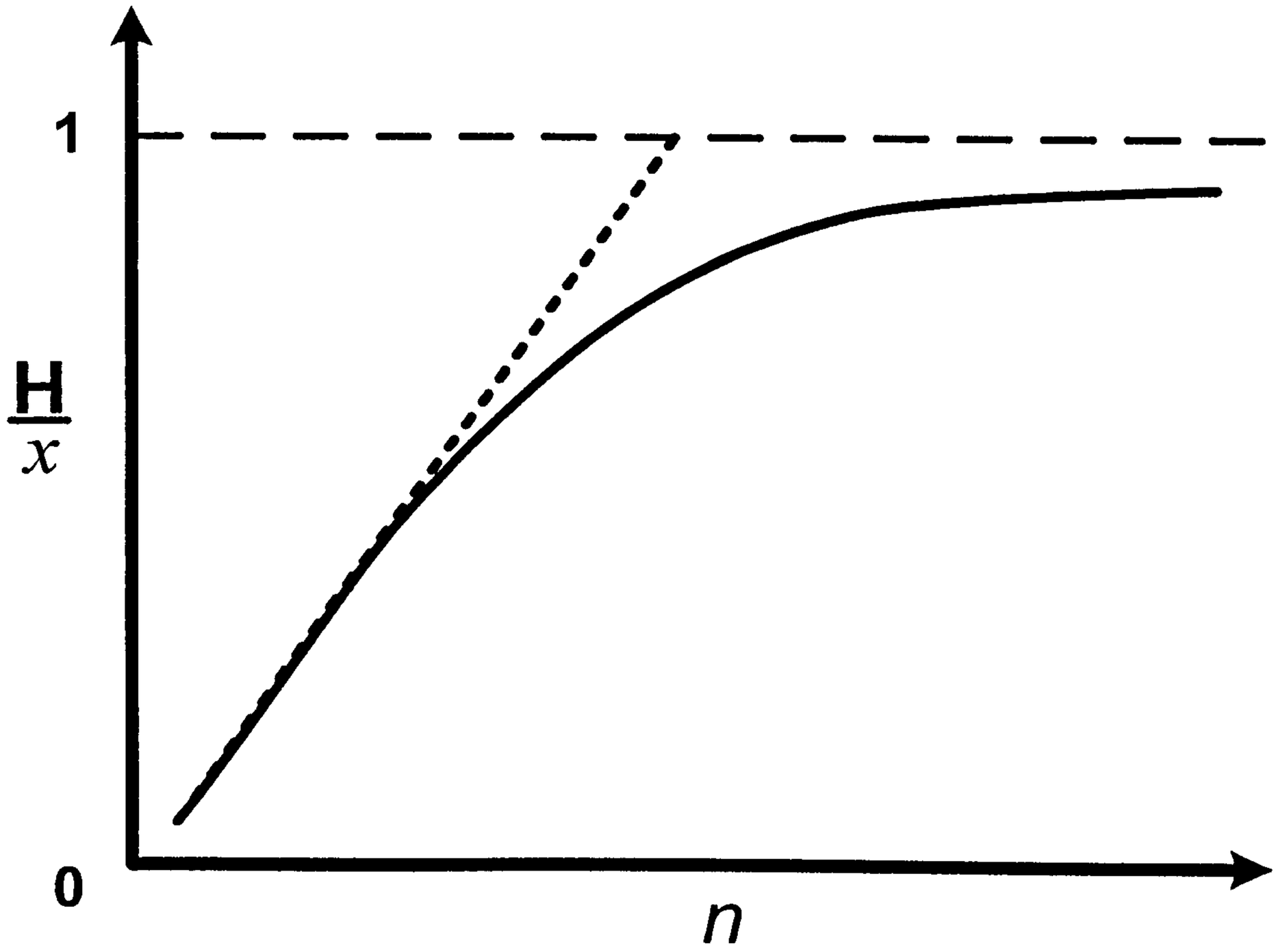

Figure 7.3 – Effect of change in *d* upon heteroplasmic saturation

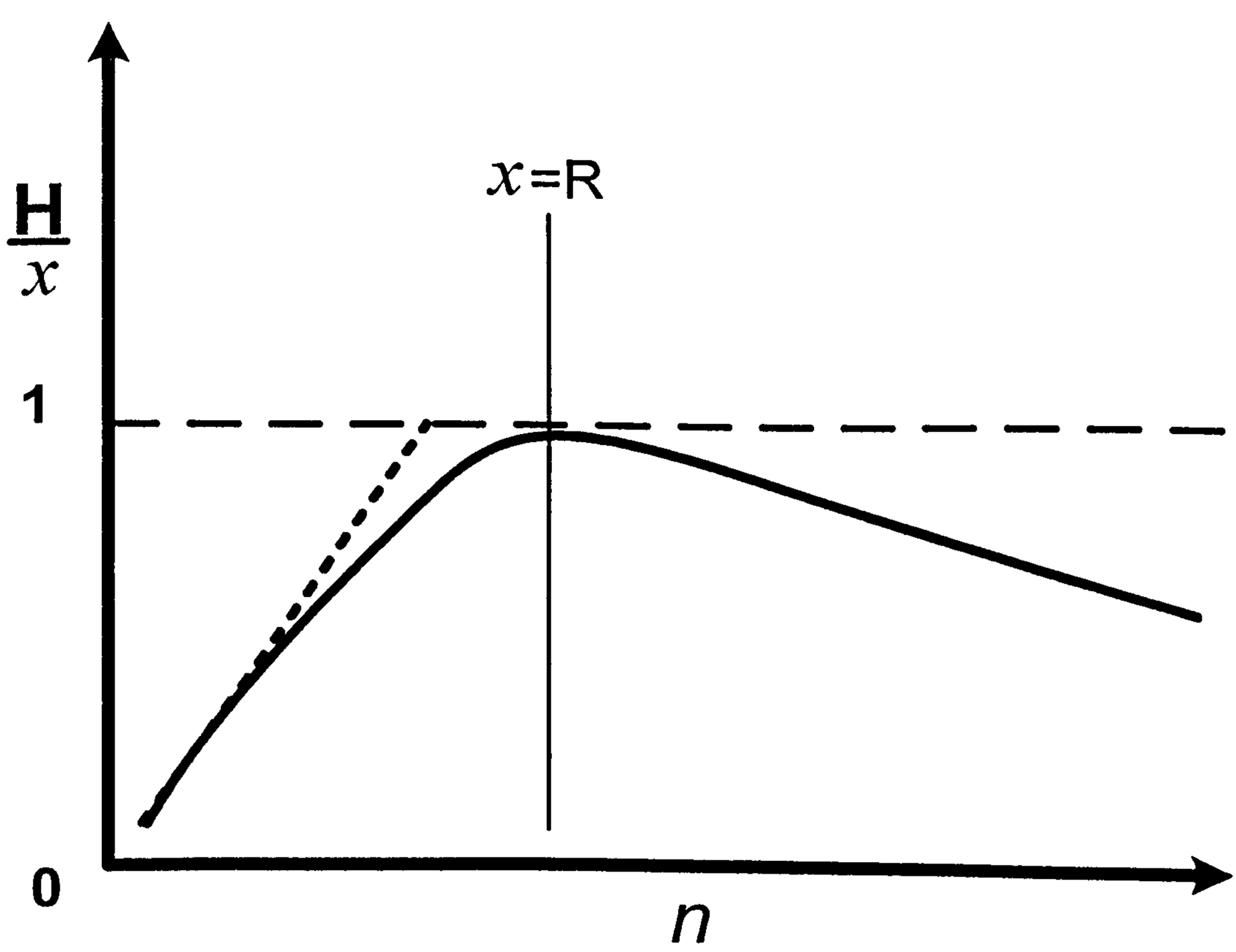

Figure 7.4 – Effect of change in *h* upon heteroplasmic saturation

7.7.2. Second iteration

The preliminary equations assume that the rate of loss of a particular DNA species by genetic drift will be unaffected as the number of DNA species tend toward heteroplasmic saturation. To combat this, a second iteration was developed.

If we introduce the term:

Q_i = Number of DNA molecules of the species *i*.

Then the proportion of a specific DNA species is determined by:

$$\frac{Q_i}{x} \qquad \textit{Equation 1.6}$$

Since:

$$x = \sum_{i=1}^{i=H} Q_i \qquad \textit{Equation 1.7}$$

Then as:

$$\frac{H}{x} \to 1, \frac{Q_i}{x} \to 0 \qquad \textit{Equation 1.8}$$

$$\Rightarrow \frac{H}{x} \propto \frac{x}{Q_i} \qquad \textit{Equation 1.9}$$

The probability of loss of a specific DNA species (*i*), by genetic drift, is proportional to the relative number of DNA molecules; that is:

$$d \propto \frac{x}{Q_i} \qquad \textit{Equation 1.10}$$

This implies that:

$$d \propto \frac{H}{x} \qquad \textit{Equation 1.11}$$

Thus:

As $\frac{H}{x} \to 1, h \to 0$ *Equation 1.12*

7.7.3. Third iteration

The preliminary and second iterations assume that the number of DNA species (H) can be infinite. Therefore we must introduce the term:

R = Maximum number of DNA species that can be produced.

Thus:

As $H \to R, h \to 0$ *Equation 1.13*

$\Rightarrow h \propto \frac{H}{R}$ *Equation 1.14*

7.7.4. Fourth iteration

The previous iterations assume that all the mutations/variations are neutral. Some DNA species, either due to their length, structure or composition, will be more likely to produce stable replications, and be less prone to mutation, than others.

If we introduce the term:

r_i = Index of neutrality

Where the index of neutrality for a given DNA species is defined by:

$r_i = \sum_{n}^{n+1} Q_i$ *Equation 1.15*

If we then compare this to the mean neutrality:

$\bar{r} = \frac{1}{H} \sum_{i=1}^{i=H} r_i$ *Equation 1.16*

Then:

$$\text{As} \quad \frac{r_i}{\bar{r}} \to 0, \frac{\partial Q_i}{\partial n} \to 0, d_i \to \infty \qquad \textit{Equation 1.17}$$

Thus, if a DNA species has an index of neutrality less than the mean, then with each subsequent replication the number of molecules of the species will reduce until removed by genetic drift. We can also show that:

$$\text{As} \quad \frac{r_i}{\bar{r}} \to \infty, \frac{\partial Q_i}{\partial n} \to \infty, H \to 1 \qquad \textit{Equation 1.18}$$

Thus if a DNA species has an index of neutrality greater than the mean then, with each subsequent replication, the number of molecules of the species will increase, until fixed by homoplasmy – the point at which r_i equals the mean as it forms the only DNA species present.

7.7.5. Heteroplasmic saturation and mtDNA segregation

The analysis suggests that a state of flux will occur in cells due to changes in mitochondrial composition. Stability will only occur if the population becomes homoplasmic, where mutations are so unstable (r=0) or that new mutations are rare or do not occur (m=0). Alternatively if two or more molecules have identical neutrality indices, centred about the mean, then a stable heteroplasmy will form. Even if there is not an increase in H (ie h=0), provided that H>1, there will be a constant change in the relative composition of mtDNA within cells/population. Even in the absence of mutation this change could be exploited for an analytical test.

The analysis predicts an event which can be described as *heteroplasmic saturation crash*. This will occur in a cell as the number of mutations approach heteroplasmic saturation. In these cells genetic drift factors are particularly evident. A cell which exceeds the point

where the number of DNA species present is half of all the DNA molecules present will, upon division, produce two daughter cells of independent genetic composition.

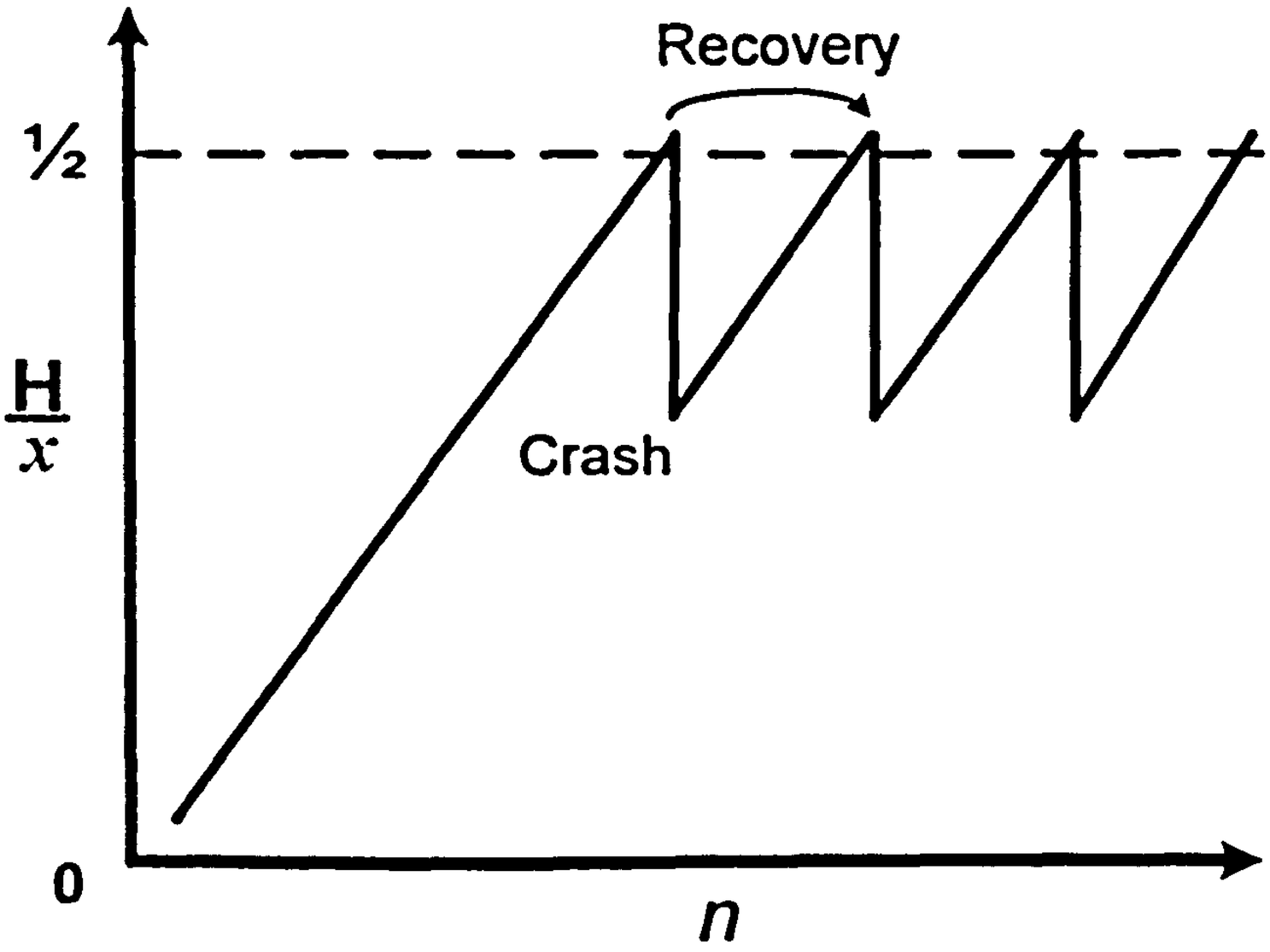

Figure 7.5 – Heteroplasmic saturation crash in division of a completely homogenous cell.

In reality it is unlikely that the cell will be completely homogenous in mtDNA composition within the cell. Segregation of mtDNA species within different mitochondrion and the relative distribution of mitochondria within the cell will allow divergence at a much lower level of heteroplasmy.

7.8. *Discussion*

The preceding equations and analysis form the basis of a preliminary model to describe the formation of heteroplasmy in eukaryotic cells due to the interaction of processes of *mutation*, *genetic drift* and *neutrality of replication*.

For a practical analysis method to be developed the following criteria must be met:

$$1 >> P(M)_D > \frac{1}{n} \qquad \textit{Equation 1.19}$$

That is;

The probability of a detectable change in the composition of a cells' mtDNA with each cell division must be significantly less than one but greater than the inverse of the number of cell divisions required in one generation.

This applies whether M is a mutation or the exploitation of segregation of heteroplasmic mtDNA species.

For a strictly mutation approach to be feasible H must be relatively low, m must be high and the newly introduced species must have neutral or greater than mean r_i. For a strictly heteroplasmic crash approach to be feasible H must be high, m must be low, h must be high and r_i of all species must approximate the mean.

Alternatively if a population can be found (or created in experimental form) where:

$$H_{individual} << H_{population}$$

With all other variables low or insignificant, then an experiment to determine oviposition site sharing may be feasible.

The empirical determination of the defined variables will allow the development of a working model from which a practical analysis protocol may be developed or, alternatively, reject the feasibility of this approach.

Chapter 8. - Conclusions

The purpose of this study was to increase the understanding of the biology of Otiorhynchus sulcatus by relating behavioural traits to oviposition and, thereby, develop an understanding of the oviposition behaviour of the vine weevil.

8.1. Problems

"It is difficult to find a black cat in a dark room – particularly if the cat is not there" Socrates.

A number of problems inherent to the study were encountered.

8.1.1. Bimodality of resource

The first difficulty in determining oviposition behaviour has been the bimodality of function. A host plant has bimodality as a source of food for the adult weevil and as a source of food for the larvae. Does the protracted presence of a vine weevil at a host plant represent a consequence of a decision, a behaviour, enacted as a consequence of the quality of the host as a food source for the adult, or as a food source for the larvae that will result from oviposition?

Surface cover, in the form of a mulch or refuge, is also a bimodal resource. A refuge can represent a place of hiding from predators, a more advantageous physical location for the adult, but at the same time represent a more advantageous physical location for ova and recently hatched larvae.

Even if bimodality represents a synergistic relationship between; the needs of the adult, the needs of the larvae resulting from oviposition, or the survival of the ova - could any

behaviour that exploits these bimodal resources be characterised as specifically oviposition behaviour?

8.1.2. Tolerance

The second difficulty in investigating oviposition behaviour of the vine weevil has been one of tolerance. *O. sulcatus* is a remarkable polyphagus insect, as an adult, and in larval form. To make an affirmative choice, a decision, requires memory – all else is sequence of encounter and tolerance.

If an organism has no discernable method of affecting an affirmative choice – if it lacks the ability to directly compare the disparate and make a decision – then behaviour can only affect outcome by specific tolerance. The vine weevil is highly tolerant and thus life history (and experimental outcome) is heavily influenced by sequence of encounter.

8.1.3. Innovation and human observation

The study has required the development of innovative techniques, the novel use of existing technology and direct observation. This approach is not conducive to firm, definitive conclusions. Firm conclusions can only be drawn by the parallax of disparate methodologies and great volumes of data produced under the blind indifference of automation.

8.2. *Technical conclusions*

A number of techniques were developed for the project. As a result of the project:

- AVOID apparatus was developed. It is now possible to investigate and plot movement of an organism without the hindrance of arena walls. Now possible to determine path lengths.

- It is feasible to track vine weevil using passive transponders.

- It is possible to extract sufficient DNA from a single vine weevil ovum to perform several PCR reactions.

8.3. *Oviposition behaviour*

Does the vine weevil exhibit oviposition behaviour? Three functional behaviour groupings were investigated:

8.3.1. *Behaviour resulting in the active positioning the ova in the direct locality of the host*

Direct observation of *Otiorhynchus sulcatus* indicates that the approach to reproduction is one of indiscriminately distributed high volume oviposition. The life history strategy is the opposite of insects such as the Ichneumonidae, which actively seek out a very specific host organism and oviposit within it, and as such exhibit a repertoire of distinct behaviour surrounding the act of oviposition. The level of site discrimination is also much less than some other insect herbivore species, such as *Sciaphilus asperatus*, that oviposit within a specific region upon the host plant (Willis, 1964). This action presumably increases the probability of survival to maturity of the oviposited clutch.

Oviposition is seemingly random and arbitrary at the micro scale – no attempt is made to oviposit in direct contact with the host plant. At the macro scale – oviposition is a function of wherever the vine weevil happens to be. The presence of ground cover greatly influences where vine weevil will take refuge during the day and consequently forms the oviposition site.

The bimodality of refuge favours the preservation of the adult (rather than quality of micro-climate for the ova). This was demonstrated when (on two separate occasions) vine weevils were found to oviposit upon silica gel granules if sufficient surface cover is present - a distinct choice of predator evasion overriding the quality of site for oviposition.

This, less than diligent approach to parenthood, can be tolerated due to the polyphagus nature and significant locomotory capabilities of the larval vine weevil. High volume indiscriminate oviposition enables the reduction to almost nil the time and action devoted to the process of finding a suitable oviposition site. This increases the available time for feeding during the night and also reduces the exposure to potential predation during the day.

Cram (1965b) observed that "[*O sulcatus*] wander randomly at night and encounter hosts fortuitously". The conclusion derived from the investigations is that *O. sulcatus* also hide in a randomly encountered, fortuitously discovered, place during the day, and oviposit indiscriminately.

8.3.2. Behaviour that is likely to keep an adult in the general vicinity of a high quality host and reduce the probability of remaining in the vicinity of a poor quality host

This could be achieved by two methods - responsive variation in locomotion or responsive variation in residency time. These behaviours were not observed. Locomotion and residency time appear to be independent of host quality.

8.3.3. Variation of clutch size in response to the quality of the host plant

Dissection of vine weevil immediately subsequent to oviposition revealed the absence of any retained ova. Oviposition for the vine weevil, therefore, appears to be an all or nothing affair. Clutch size is variable – but appears to be purely a physiological response to the availability of nutrients.

Behaviour, under conditions of choice, appears to preclude the oviposition upon host plants of high in direct proportion to the plant's ability to supply nutrients. Clutch size appears to be a physiological reaction to physical and nutritional conditions.

8.3.4. Indiscriminate oviposition

Oviposition appears to be a function of locomotion of the adult weevil and the accumulation of nutrients acquired from previously encountered hosts – a life history strategy tolerantly enabled by exceptional polyphagy, large clutch sizes and dispersal via larval locomotion.

This tentative conclusion constitutes a veritable absence of a specific behaviour related to oviposition. The conclusion is drawn more from an absence of observable variation in behaviour rather than specific observation. Although it is possible that the experiments conducted simply failed to record a specific behavioural pattern – although "absence of

proof is not proof of absence" - the absence of an observation of a specific behavioural pattern, coupled with the eminent sensibility of the life history strategy is highly suggestive of this conclusion.

References and Bibliography

1. Alberts, B, Bray, D, Lewis, J, Raff, M, Roberts, K and Watson, J D. 1989. *Molecular Biology of the Cell.* Second. Garland Publishing, 136 Madison Avenue, New York, NY. 10016. 1219.

2. Alford, D V. 1996. Adult vine weevil activity in black currant plantations in South West England *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-Verlag GmbH BerlinlWein Kurfurstendamm 57, d-1O707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 31-35.

3. Alonso, J C, Alonso, J A, Bautista, L M, Munoz-Pulidor, R. 1995. Patch use in cranes: A field test of optimal foraging theory. *Animal Behaviour.* 49, 1367-1379.

4. Anon. 1974. Commonwealth Institute of Entomology Distribution maps of pests. Series A (Agricultural), Map 331. Pest: *Otiorhynchus sulcatus.*

5. Anon. 1996a. Nursery claims over vine weevil control. *Grower.* 22 August 1996, 5.

6. Anon. 1996b. Timing the nematode strike. *Grower.* 15 August 1996, 14-15.

7. Baatrup, E and Bayley, M. 1993. Quantitative analysis of spider locomotion employing computer-automated video tracking. *Physiology and Behaviour.* 54, 83-90.

8. Backhaus, G F. 1996. Vine weevil problems on ornamental plants. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-Verlag GmbH BerlinlWein Kurfurstendamm 57, d-l0707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 12-18.

9. Bathon, H. 1996. Selection and use of entomopathogenic nematodes against vine weevil. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts- V erlag GmbH BerlinlWein Kurfurstendamm 57, d-l0707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 81-85.

10. Bergman, C. M., Schaefer, J. A. and Luttich, S. N. (2000) Caribou movement as a correlated random walk. *Oecologia,* **123,** 364-374.

11. Blackshaw, R P. 1983. Tests of insecticides against vine weevil larvae. *Annals Applied Biology.* **102** (supplement), 36-37.

12. Blackshaw, R P. 1984. Studies on the chemical control of vine weevil larvae. *British Crop Protection Conference - Pests and diseases.* 1093-1098.

13. Blackshaw, R P. 1986. An evaluation of seven controlled-release insecticides for the control of vine weevil larvae. *Journal of Horticultural Science.* **61,** 109-111.

14. Blackshaw, R P. 1987. Studies on the chemical control of vine weevil larvae in hardy-ornamental nursery stock. *Journal of Horticultural Science.* **62,** 63-66.

15. Blackshaw, R P. 1992. Black vine weevil *(Otiorhynchus sulcatus* (F.)) oviposition on polyanthus plants outdoors in Northern Ireland. *Journal of Horticultural Science.* **76,** 641-646.

16. Blackshaw R P. 1996b. Importance of overwintering adults to summer oviposition in Northern Ireland. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-Verlag GmbH BerlinlWein Kurfurstendamm 57, d-l0707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 36-40.

17. Blackshaw, R P and O'Neill, S. 1987. Chlorpyrifos compost treatment for vine weevil control: growth of ornamental plants compared with an aldrin standard. *Journal of Horticultural Science.* **62,** 67-69.

18. Blackshaw, R P and Thompson, D. 1993. Comparative effects of bark and peat based composts on the occurance of vine weevil larvae and the growth of containerized polyanthus. *Journal of Horticultural Science.* **68,** 725-729.

19. Borek, V , Elberson, L R, McCaffrey, J P and Morra, M J. 1995. Toxicity of aliphatic and aromatic isothiocyanates to eggs of the black vine weevil (COLEOPTERA, CURCULIONIDAE). *Journal of Economic Entomology.* **88,** 1192-1196.

20. Bouche, P F. 1834. Naturgeschichte der insecten besonders in hinsicht ihrer erstfn zustande als larven und puppen. 216p. Berlin.

21. Boyce, T M, Zwick, M E and Aquadro, C F. 1989. Mitochondrial DNA in the bark weevils: Size, structure and heteroplasmy. *Genetics.* **123,** 825-836.

22. Bradford, M. M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248

23. Budaev, S. V. (1997) 'Personality' in the guppy (*Poecilia reticulata*): a correlation study of exploratory behaviour and social tendency. *Journal of Comparative Psychology*, **111**, 399-411.

24. Buxton, J H. 1996. Current status of vine weevil as a pest in the UK. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts- V erlag GmbH BerlinlW ein Kurfurstendamm 57, d-1O707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 6-11.

25. Byers, J. A. (2001) Correlated random walk equations of animal dispersal resolved by simulation. *Ecology,* **82,** 1680-1690.

26. Charnov, E. L. 1976. Optimal foraging, the marginal value theorem. Theor. Pop. Biol., **9**, 129-136

27. Coleman, K. and Wilson, D. S. (1998) Shyness and boldness in pumpkinseed sunfish: individual differences are context specific. *Animal Behaviour*, **56**, 927-936.

28. Cone, W W. 1963. The black vine weevil, *Brachyrhinus*[sic] *sulcatus*, as a pest of grapes in South Central Washington. *Journal of Economic Entomology.* **56**, 677-680.

29. Cone, W W. 1968. Black vine weevil larval damage to concord grape roots at different population densities. *Journal of Economic Entomology.* **61,** 1220-1224.

30. Cram, W T. 1964. Fecundity of the root weevils *Brachyrhinus* [sic] *sulcatus* and *Sciopithes obscurus* on strawberry in the laboratory and outdoors. *Canadian Journal of Plant Science.* **45**, 169-176.

31. Cram, W T. 1965a. Fecundity of the root weevils *Brachyrhinus sulcatus* and *Sciopithes obscurus* on strawberry at different conditions of host plant nutrition. *Canadian Journal of Plant Science.* **45,** 219-225.

32. Cram, W T. 1965b. Fecundity of the black vine weevil on strawberry with nitrogen supplied in the ammonium or nitrate form. *Canadian Journal of Plant Science.* **45**, 295-296.

33. Cram, W T. 1980. Fecundity of the black vine weevil, Otiorhynchus sulcatus (Coleoptera: Curculionidae), fed foliage from some current cultivars and advanced selections of strawberry in British Columbia. *Journal of the Entomological Society of British Columbia.* **77**, 25-26.

34. Cram, W T and Daubeny, H A, 1982, Responses of black vine weevil adults fed foliage from genotypes of strawberry, red raspberry and red raspberry-blackberry hybrids. *Hortscience.* **17**, 771-773.

35. Cram, W T and Pearson, W D. 1965. Fecundity of the black vine weevil *(Brachyrhinus* [sic] *sulcatus* (F.) fed on foliage of blueberry, cranberry and weeds from peat bogs. *Proceedings of the Entomological Society of Columbia.* **62**, 25-27.

36. Doss, R P and Shanks, C H. 1984. Black vine weevil, *Otiorhynchus-sulcatus* (Coleoptera, Curculionidae), phagostimulants from alpine strawberry. *Environmental* entomology, **13**, 691-695.

37. Doss, R P and Shanks, C H. 1988. The influence of leaf pubescence on the resistance of selected clones of beach strawberry (*fragaria-chiloensis* (L) duchesne) to adult black vine weevils (*Otiorhynchus-sulcatus* f). *Scientia Horticulturae.* **34**, 47-54.

38. Doss, RP, Shanks, CH, Chamberlain, J D and Garth, J K L. 1987. Role of leaf hairs in resistance of a clone of beach strawberry, *fragarta-chiloensis*, to feeding by adult black vine weevil, *Otiorhynchus sulcatus* (Coleoptera, Curculionidae). *Environmental Entomology.* **16**, 764-768.

39. Doss, R P, Shanks, C H, Sjulin, T M And Garth, J K L. 1991. Evaluation of some *fragaria-chiloensis* x (f x ananasa) seedlings for resistance to black vine weevil. *Scientia Horticulturae.* **48**, 233-239.

40. Doss, RP and Shanks, C H (Jr). 1985. Effect of age on the feeding pattern of the adult black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Annals of the Entomological Society of America.* **78**,322-325.

41. Evenhuis, H H. 1978. Bionomics and control of the black vine weevil *Otiorhynchus sulcatus. Med Fac Landbouww Rijksuniv Gent.* **43**, 607-611.

42. Feytaud, J. 1918. Etude sur l'otiorhynque sillonne *(Otiorhynchus sulcatus Fabr.). Annales du Service des Epiphyties.* **5**, 145-192.

43. Fox, C W and Tatar, M. 1994. Oviposition substrate affects adult mortality, independent of reproduction, in the seed beetle *Callosobruchus maculatus. Ecological Entomology.* **19**, 108-110.

44. Frers, A. 1996. Casual experiences of vine weevil biology before, during and after carrying out control tests. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell (Wissenschafts-Verlag GmbH BerlinlWein Kurfurstendamm 57, d-1O707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 46-50.

45. Gange, A C. 1996. Reduction in vine weevil larval growth by mycorrhizal fungi. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-V erlag GmbH BerlinlWein Kurfurstendamm 57, d-1O707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 56-60.

46. Garth, G S and Shanks, C H (Jr). 1978. Some factors affecting infestation of strawberry fields by the black vine weevil in Westem Washington. *Journal of Economic Entomology.* 71, 443-448.

47. Hanula, J L. 1988. Oviposition preference and host recognition by the black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Environmental Entomology.* 17, 694-698.

48. Hanula, J L. 1990. Monitoring adult emergence, ovary maturation and control of the black vine weevil (Coleoptera: Curculionidae). *Journal of Entomological Science.* 25, 134-142.

49. Hanula, J L. 1993. Vertical distribution of black vine weevil (Coleoptera: Curculionidae) immatures and infection by entomogenous nematodes in soil columns and field soil. *Journal of Economic Entomology.* 86,340-347.

50. Harrison, R G, Rand, D M and Wheeler, W C. 1985. Mitochondrial DNA size variation within individual crickets. *Science.* 228, 1446-1448.

51. Hawkins, J D. 1996. *Gene structure and expression.* 3. Cambridge University Press, Cambridge. 212.

52. Hesjedal, K. 1984. Influence of the nitrogen-content in strawberry leaves on the fecundity of the vine weevil, *Otiorhynchus sulcatus* f (Coleoptera, Curculionidae). *Acta Agricultura Scandinavica.* **34**, 188-192.

53. Hibbard, B E, Higdon, M L, Duran, D P, Schweikert, Y M, Ellersieck, M R. 2004. Role of egg density on establishment and plant-to-plant movement by western corn rootworm larvae (Coleoptera: Chrysomelidae). *Journal of Economic Entomology.* **97**, 871-882.

54. Iguchi, K., Matsubara, N. and Hakoyama, H. (2001) Behavioural individuality assessed from two strains of cloned fish. *Animal Behaviour*, **61**, 351-356.

55. Janz, N, Nylin, S. 1997. The role of female search behaviour in determining host plant range in plant feeding insects: a test of the information processing hypothesis. *Proceedings: Biological SciencesI.* **264**. 701-707.

56. Janz, N. 2003. The cost of polyphagy: oviposition decision time vs error rate in a butterfly. *Oikos*, **100(3)**, 493-496.

57. Johnson, S. (2001) *Emergence* The Penguin Press, London 288 pp.

58. Kareiva, P. M. and Shigesada, N. (1983) Analyzing insect movement as a correlated random-walk *Oecologia,* **56,** 234-238.

59. Kingsley, RI. 1898. On the occurrence of the black vine weevil *(Otiorhynchus sulcatus)* in Nelson. *Transactions and Proceedings of the New Zealand Institute.* **22**, 338-340.

60. Labuschagne, L. 1994. Pest status, biology and behaviour *of* vine weevil. *Vine Weevil workshop Conference Proceedings.* 5-12.

61. LaLone, R S and Clarke, R G. 1981. Larval development *of Otiorhynchus sulcatus* (Coleoptera: Curculionidae) and effects of larval density on larval mortality and injury to rhododendron. *Environmental Entomology.* **10**, 190-191.

62. Maier, C T. 1983. Use of trap-boards for detecting adults of the black vine weevil, *Otiorhynchus-sulcatus* (fabricius) (Coleoptera, Curculionidae). *Proceedings* Of *The Entomological Society* of *Washington,* , .

63. Maier, C T. 1981. Reproductive success *of* the black vine weevil, *Otiorhynchus sulcatus* (F.) fed different foliar diets and evaluation *of* techniques for breeding. *Environmental Entomology.* **10**, 928-932.

64. Masaki, M, Ohmura, K and Ichinohe, F. 1984. Host range studies *of* the black vine weevil, *Otiorhynchus sulcatus* (FABRICIUS) (Coleoptera:Curculionidae). *Applied Entomology* and *Zoology.* **19**, 95-106.

65. Mason, E C. 1960. Observations on the life history and control *of* the vine weevil on cyclamen and foliage plants. *Plant Pathology.* **9**, 29-33.

66. May, P D and Ellis, G AV. 1996. The development *of* controlled-release technology to give long-term control *of* vine weevil larvae in omamentals. *Second international workshop* on vine weevil *(Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-Verlag GmbH Berlin/WeinKurfurstendamm 57, d-l0707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 118-122.

67. May, R. M. (1976) Simple mathematical models with very complicated dynamics. *Nature*, **261**, 459-467.

68. Montgomery, M E and Nielsen, D G. 1979. Embryonic development of

Otiorhynchus sulcatus: effect of temperature and humidity. *Entomologia experimentalis et applicata.* **26**, 24-32.

69. Moorhouse, E R. 1990. *The potential of the entomogenous fungus Metarhizium anisopliae as a microbial control of the black vine weevil, Otiorhynchus sulcatus.* PhD University of Bath, Bath.

70. Moorhouse, E R, Charnley, A K and Gillespie, A T. 1992a. A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Annals of Applied Biology.* **121**, 431-454.

71. Moorhouse, E R, Fenlon, J S, Gillespie, A T and Charnley, A K. 1992b. Observations on the development, oviposition and fecundity of vine weevil adults, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae). *Entomologist's Gazette.* **43**, 207-218.

72. Moorhouse, E R, Gillespie, A T and Charnley, A K. 1994. The influence of temperature on the susceptibility of vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae), larvae to *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Annals of Applied Biology.* **124**, 185-193.

73. Morales, J. M. and Ellner, S. P. (2002) Scaling up animal movements in heterogeneous landscapes: The importance of behavior *Ecology,* **83**, 2240-2247.

74. Morgan, D. 1996. Modelling vine weevil population dynamics. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts- V erlag GmbH Berlin/WeinKurfurstendamm 57, d-l0707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 51-55.

75. Moritz, C, Dowling, T E and Brown, WM. 1987. Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. *Annual Review of Ecology and Systematics.* **18**, 269-292.

76. Mosson, H J, Watkins, R W and Edwards, J P. 1996. The cinnamic acid derivative cinnamamide as a repellent against the vine weevil *Otiorhynchus sulcatus* (Coleoptera:Curculionidae). *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-Verlag GmbH BerlinlWein Kurfurstendamm 57, d-1O707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 95-100.

77. Neiswander, R B. 1953. Control of the black vine weevil. *Journal of Economic Entomology.* **46**, 234-237.

78. Neubauer, C H R. 1996. Biological control of *Otiorhynchus sulcatus* with *Steinernema* nematodes, under field conditions in northern Germany. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts- V erlag GmbH Berlin/Wein Kurfurstendamm 57, d-1O707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 91-94.

79. Nielsen, D G and Dunlap, M J. 1981. Black vine weevil: Reproductive potential on selected plants. *Annals of the Entomological Society of America.* **74**, 60-65.

80. Nielsen, D G and Roth, J R. 1985. Influence of potting media on toxicity of bendiocarb and carbofuran to first-instar black vine weevil (Coleoptera, Curculionidae). *Journal of Economic Entomology.* **78**, 742-747.

81. Nylin, S, Bergstrom, A, Janz, N. 2000. Butterfly host plant choice in the face of possible confusion. *Journal of Insect behaviour.* **13(4)**, 469-482.

82. Ochs, G. 1960. Tests on the transmission of three viruses of vines by mites and insects. *Zeitschriftfur Angewandte Zoologie.* **47**, 485-491.

83. Penman, D Rand Scott, R R. 1976. Adult emergence and egg production of the black vine weevil in Canterbury. *New Zealand Journal of Experimental Agriculture.* **4**, 385-389.

84. Pickett, J A, Bartlet, E, Buxton, J H, Wadhams, L J and Woodcock, C M. 1996. Chemical ecology of adult vine weevil. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-Verlag GmbH Berlin/Wein Kurfurstendamm 57, d-1O707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 41-45.

85. Pires C S S, Price, P W, Fontes, E G. 2000. Preference-performance linkage in the neotropical spittlebug *Deois flavopicta,* and its relation to the Phylogenetic Contraints Hypothesis. *Ecological Entomology.* **25**, 71-80.

86. Prado, C E. 1988. Notes on some insects of agricultural importance in Chile. *Agricultura Technica.* **48**, 51-54.

87. Primrose, S B. 1995. *Principles of Genome Analysis.* Blackwell Science. 148.

88. Rand, D M, Dorfsman, M and Kann, L M. 1994. Neutral and non-neutral evolution of *Drosphila* mitochondrial DNA. *Genetics.* **138**, 741-756.

89. Rand, D M and Harrison, R G. 1986. Mitochondrial DNA transmission genetics in crickets. *Genetics.* **114**, 955-970.

90. Reale, D., Gallant, B. Y., Leblanc, M. and Festa-Bianchet, M. (2000) Consistency of temperament in bighorn ewes and correlates with behaviour and life history. *Animal Behaviour*, **60**, 589-597.

91. Sambrook, J, Fritsch, E F, Maniatis, T. 1987. Molecular Cloning: A laboratory manual. Third. Cold Spring Harbor Laboratory Press. 2344.

92. Shanks, C H, Chase, D L and Chamberlain, J D. 1984. Resistance of clones of wild strawberry (frageria-chiloensis) to adult *Otiorhynchus-sulcatus* and *Otiorhynchus-ovatus* (Coleoptera, Curculionidae), *Environmental Entomology*. **13**, 1042-1045.

93. Shanks, C H (Jr). 1980. Strawberry and yew as hosts of adult black vine weevil and effects on oviposition and development of progeny. *Environmental Entomology*. **9**, 530-532.

94. Shanks, C H (Jr). 1981. Root weevils on berry crops. *Washington State University Cooperative Extension Bulletin, No 0965.*

95. Shanks, C H (Jr) and Doss, R P. 1986. Black vine weevil (Coleoptera: Curculionidae) feeding and oviposition on leaves of weevil-resistant and -susceptible strawberry clones presented in various quantities. *Environmental Entomology*. **15**, 1074-1077.

96. Shanks, CH (Jr) and Finnigan, B F. 1973. Temperature and relative humidity effects on eggs and first-stage larvae of the black vine weevil, *Otiorhynchus sulcatus. Environmental Entomology.*

97. Schiers, J, Zoebisch, T G, Schuster, D J, De Bruyn, L. 2004. Optimal foraging shapes host preferences of a polyphagus leafminer. *Ecological Entomology*. **29**, 375-379.

98. Schiers, J, Bruyn, L D, Verhagen, R. Seasonal changes in leaf nutritional quality influence grass miner performance. *Ecological Entomology*. **27**, 84-93.

99. Singer, M C, Vasco, D, Parmesan, C, Thomas, C D, and Ng, D. 1992. Distinguishing between 'preference' and 'motivation' in food choice: example from insect oviposition. *Animal Behaviour.* 44, 463-471.

100. Smith, F F. 1932. Biology and control of the black vine weevil. *USDA Technical Bulletin.* **325**, 1-45.

101. Solignac, M, Genermont, J, Monnerot, M and Mounolou, J C. 1984. Genetics of mitochondria in *Drosophila:* mtDNA inheritance in heteroplasmic strains of *D.*

mauritiana. Mol Gen Genet. **197**, 183-188.

102. Solignac, M, Genermont, J, Monnerot, M and Mounolou, J C. 1987. Drosophila mitochondrial genetics: Evolution of heteroplasmy through germ line cell divisions. *Genetics.* **117**, 687-696.

103. Solignac, M, Monnerot, M and Mounolou, J C. 1986b. Mitochondrial DNA evolution in the *IfManogaster* species subgroup of *Drosophila. Journal of Molecular Evolution.* **23**, 31-40.

104. Steinbauer, M J. 1999. The population ecology of *Amorbus dallas* (Hemiptera: Coreidae) species in Australia. *Entomologia Experimentalis et Applicata.* **91**, 175-182.

105. Stenseth, C. 1979. Effects of temperature on development of *Otiorrhynchus* [sic] *sulcatus* (Coleoptera: Curculionidae). *Annals of Applied Biology.* **91**, 179-185.

106. Stimmann, M W, Kaya, H K, Burlando, T M and Studdert, J P. 1985. Black vine weevil management in nursery plants. *California Agriculture.* January-February 1985, 25-26.

107. Stinner, RE and Bacheler, J E. 1993. The myth of random movement - old concept with a new twist. *Journal of Agricultural Entomology.* **10**, 247-265.

108. van Emden, F. ????. Eggs, egg-laying habits and larvae of short-nosed weevils. *Eighth International Congress of Entomology.* 365-372.

109. Van Tol, R W H M. 1996a. Prospects for biological control of black vine weevil *(Otiorhynchus sulcatus*) in nursery stock. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-Verlag GmbH BerlinlWein Kurfurstendamm 57, d-10707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 69-75.

110. Van Tol, R W H M. 1996b. A strategy for control of black vine weevil *(Otiorhynchus sulcatus)* in integrated pest management programme in nursery stock. *Second international workshop* on *vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-V erlag GmbH Berlin/Wein Kurfurstendamm 57, d-l0707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 76-80.

111. Van Tol, R W H M, and Visser, J H, and Sabelis, M W. 2002. Olfactory responses of the vine weevil, *Otiorhynchus sulcatus*, to tree odours. *Physiological Entomology.* **27** 213-222.

112. Van Tol, R W H M, and Visser, J H. 2002. Olfactory antennal responses of the vine weevil *Otiorhynchus sulcatus* to plant volatiles. *Entomologia Experimentalis et Applicata*. **102**. 49-64

113. Vlad, M O. 1994. A random walk approach to spiral motion. *Journal of Physics A - Mathematical and General.* **27**, 1791-1810.

114. Vogt Heidrun. 1996. Vine weevil problems in food crops. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-Verlag GmbH Berlin/Wein Kurfurstendamm 57, d-1O707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 19-23.

115. Warner, RE and Negley, F B. 1976. The genus *Otiorhynchus* in America north of Mexico (Coleoptera: Curculionidae). *Proceedings of the Entomological Society of Washington.* **78**, 240-262.

116. Westwood, J O. 1837. A series of articles on the insects most injurious to cultivators, No. 2. The natural history of a weevil, which is very destructive to collections of succulent plants. *Gardener's Magazine.* **13**, 157-161.

117. Willis, R J. 1964. *The bionomics and larval morphology of the Otiorhynchid* pests of soft fruit crops. PhD, Queen's University of Belfast., Belfast.

118. Wu, H., Li, B. L., Springer, T. A. and Neill, W. H. (2000) Modeling animal movement as a persistent random walk in two dimensions: expected -magnitude of net displacement *Ecological Modelling,* **132,** 115-124

119. Zepp, D B, Dierks, A Z and Sanders, DJ. 1979. Effects of diflubenzuron on black vine weevil oviposition, egg viability, and adult longevity. *Journal of the Kansas Entomological Society.* **52,** 662-666.

120. Zimmermann, G. 1996. Microbial control of vine weevil. *Second international workshop on vine weevil (Otiorhynchus sulcatus Fabr.) (Coleoptera: Curculionidae). Conference Proceedings.* Blackwell Wissenschafts-Verlag GmbH Berlin/Wein Kurfurstendamm 57, d-l0707 Berlin. Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. 64-68.

Appendix I – Modified Long Ashton Formula

Solution	Content	Concentration
1	$MgSO_4.7H_2O$	373mM
2	$MnSO_4.4H_2O$	10mM
	$ZnSO_4$	1mM
	$CuSO_4.5H_2O$	1mM
	$Na_2MoO_4.2H_2O$	0.5mM
	NaCl	100mM
	$CoSO_4.7H_2O$	0.2mM
	H_3BO_3	60mM
3	KNO_3	500mM
	$Ca(NO_3)_2$	490mM
4	$NaH_2PO_4.2H_2O$	333mM
5	$CaSO_4.2H_2O$	Insoluble
6	K_2SO_4	375mM
7	FeNa EDTA	9mM

Table I.1 – Constituent solutions for the modified Long Ashton formula

%N	1	2	3	4	5	6	7
0%	8	1	0	4	0.72	16	5
10%	8	1	0.8	4	0.648	14.4	5
20%	8	1	1.6	4	0.576	12.8	5
30%	8	1	2.4	4	0.504	11.2	5
40%	8	1	3.2	4	0.432	9.6	5
50%	8	1	4	4	0.36	8	5
60%	8	1	4.8	4	0.288	6.4	5
70%	8	1	5.6	4	0.216	4.8	5
80%	8	1	6.4	4	0.144	3.2	5
90%	8	1	7.2	4	0.072	1.6	5
100%	8	1	8	4	0	0	5

Table I.2 – Volume of constituent solution used in $ml.l^{-1}$ to make the modified N depleted Long Ashton formula (except 5 which is added in powder form as $g.l^{-1}$).

Appendix II – Key to path diagrams from AVOID experiments

Experiment 1

run	Group	group name	experiment	replication	Weevil
1	1	Pre-oviposition	1	1	1
2	1	Pre-oviposition	1	1	2
3	1	Pre-oviposition	1	1	3
4	1	Pre-oviposition	1	1	4
5	1	Pre-oviposition	1	1	5
6	1	Pre-oviposition	1	1	6
7	1	Pre-oviposition	1	2	1
8	1	Pre-oviposition	1	2	2
9	1	Pre-oviposition	1	2	3
10	1	Pre-oviposition	1	2	4
11	1	Pre-oviposition	1	2	5
12	1	Pre-oviposition	1	2	6
13	2	Post-oviposition	1	1	1
14	2	Post-oviposition	1	1	2
15	2	Post-oviposition	1	1	3
16	2	Post-oviposition	1	1	4
17	2	Post-oviposition	1	1	5
18	2	Post-oviposition	1	1	6
19	2	Post-oviposition	1	2	1
20	2	Post-oviposition	1	2	2
21	2	Post-oviposition	1	2	3
22	2	Post-oviposition	1	2	4
23	2	Post-oviposition	1	2	5
24	2	Post-oviposition	1	2	6

Experiment 2

run	Group	group name	experiment	replication	Weevil
25	3	No food	2	1	7
26	3	No food	2	1	8
27	3	No food	2	1	9
28	3	No food	2	1	10
29	3	No food	2	1	11
30	3	No food	2	1	12
31	3	No food	2	2	7
32	3	No food	2	2	8
33	3	No food	2	2	9
34	3	No food	2	2	10
35	3	No food	2	2	11
36	3	No food	2	2	12
37	4	Low N diet	2	1	13
38	4	Low N diet	2	1	14
39	4	Low N diet	2	1	15
40	4	Low N diet	2	1	16
41	4	Low N diet	2	1	17
42	4	Low N diet	2	1	18
43	4	Low N diet	2	2	13
44	4	Low N diet	2	2	14
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46	4	Low N diet	2	2	16
47	4	Low N diet	2	2	17
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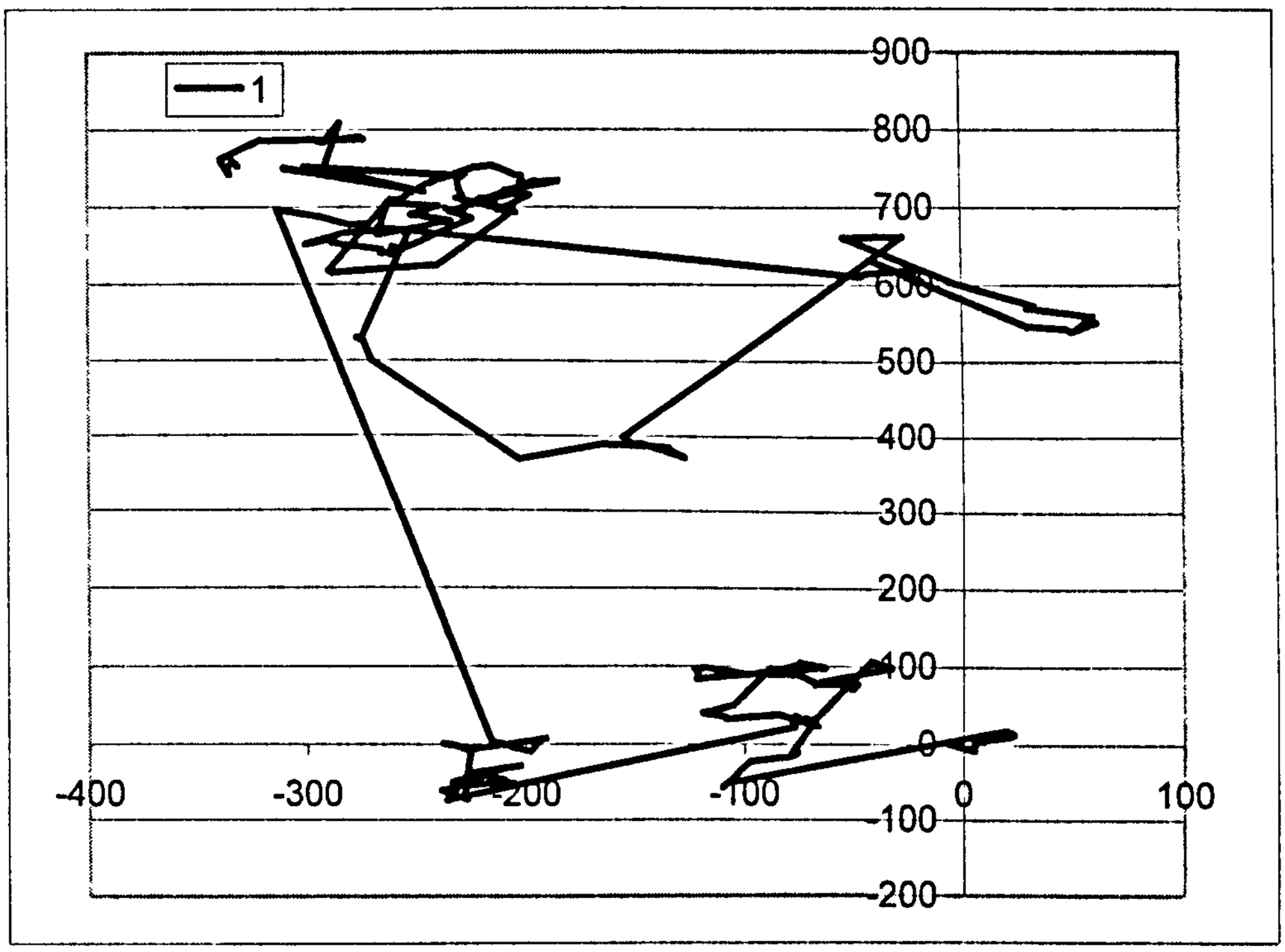
53	5	High N Diet	2	1	23
54	5	High N Diet	2	1	24
55	5	High N Diet	2	2	19
56	5	High N Diet	2	2	20
57	5	High N Diet	2	2	21
58	5	High N Diet	2	2	22
59	5	High N Diet	2	2	23
60	5	High N Diet	2	2	24

Experiment 3

run	Group	group name	experiment	replication	Weevil
61	6	Grouped	3	1	25
62	6	Grouped	3	1	26
63	6	Grouped	3	1	27
64	6	Grouped	3	1	28
65	6	Grouped	3	1	29
66	6	Grouped	3	1	30
67	6	Grouped	3	2	25
68	6	Grouped	3	2	26
69	6	Grouped	3	2	27
70	6	Grouped	3	2	28
71	6	Grouped	3	2	29
72	6	Grouped	3	2	30
73	7	Un-grouped	3	1	31
74	7	Un-grouped	3	1	32
75	7	Un-grouped	3	1	33
76	7	Un-grouped	3	1	34
77	7	Un-grouped	3	1	35
78	7	Un-grouped	3	1	36
79	7	Un-grouped	3	2	31
80	7	Un-grouped	3	2	32
81	7	Un-grouped	3	2	33
82	7	Un-grouped	3	2	34
83	7	Un-grouped	3	2	35
84	7	Un-grouped	3	2	36

Appendix III – Path diagrams from AVOID experiments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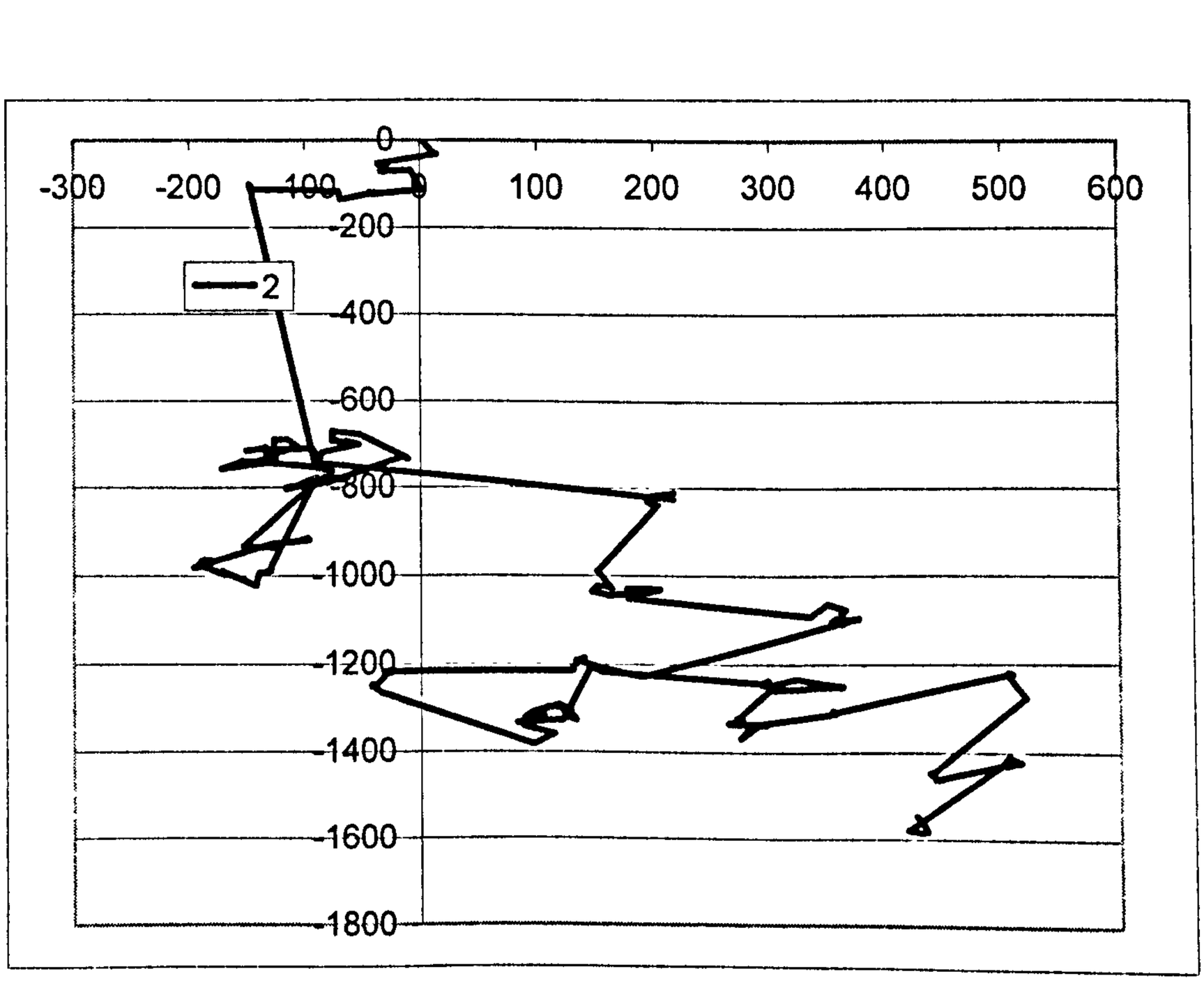
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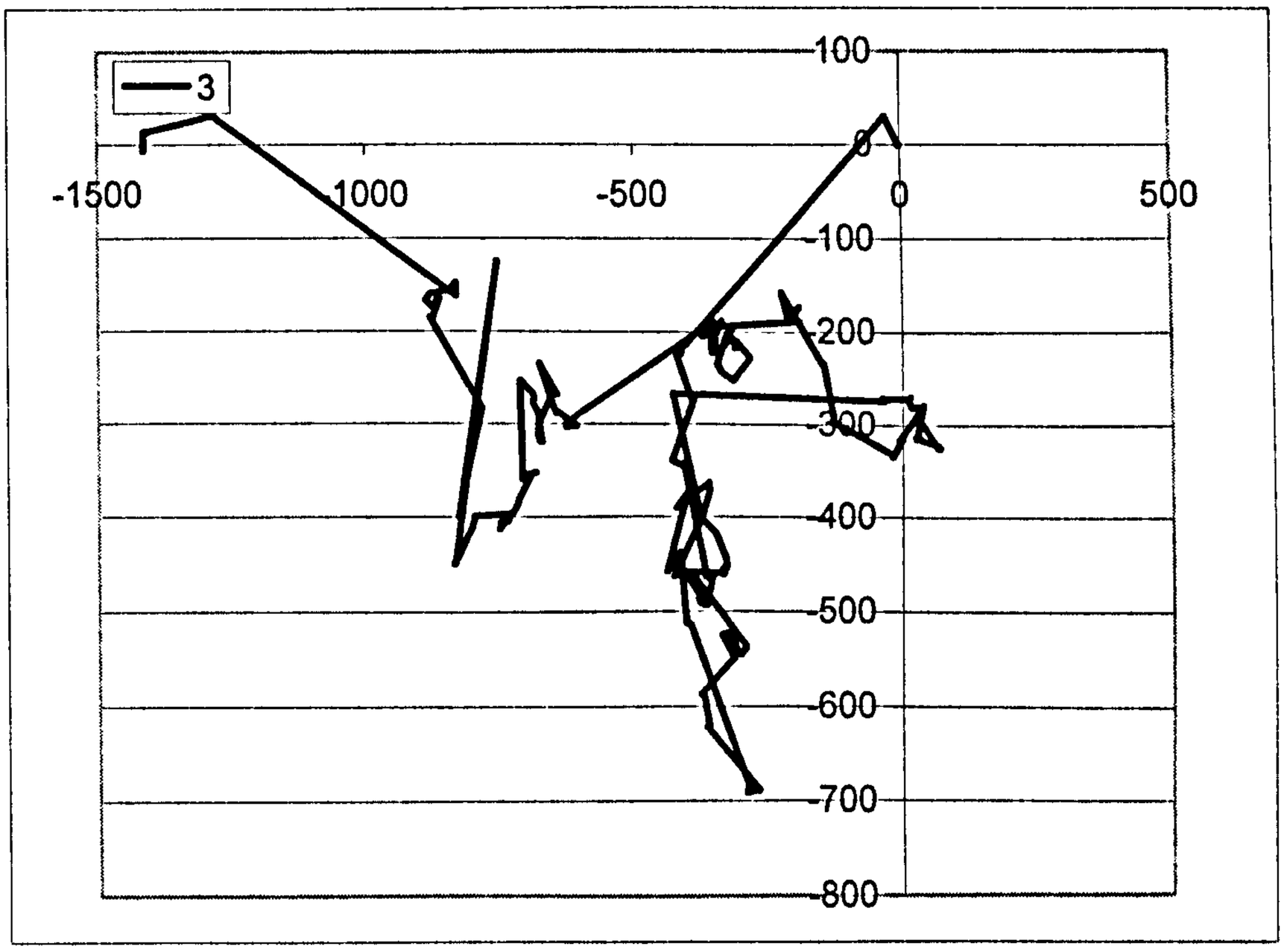
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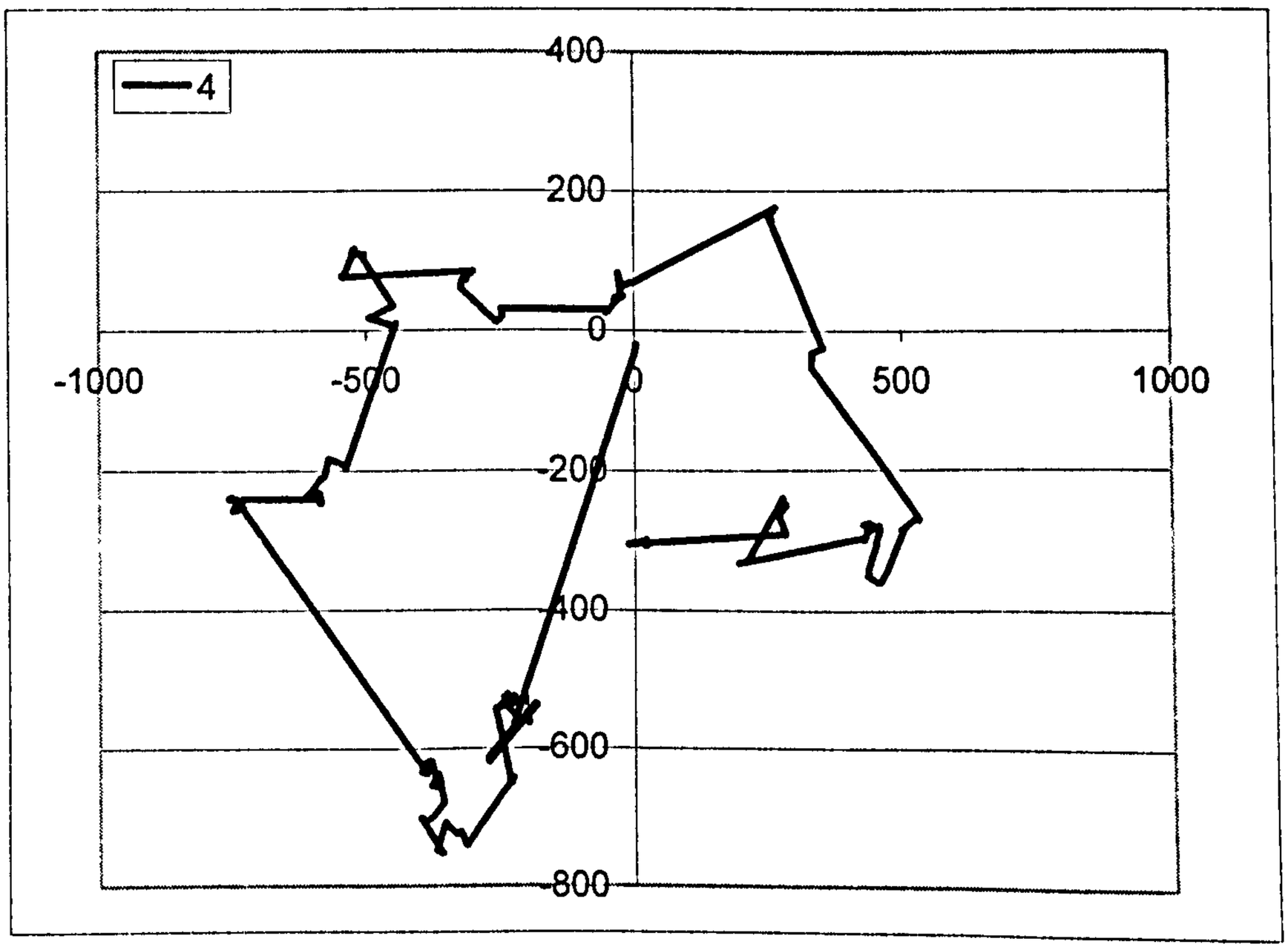
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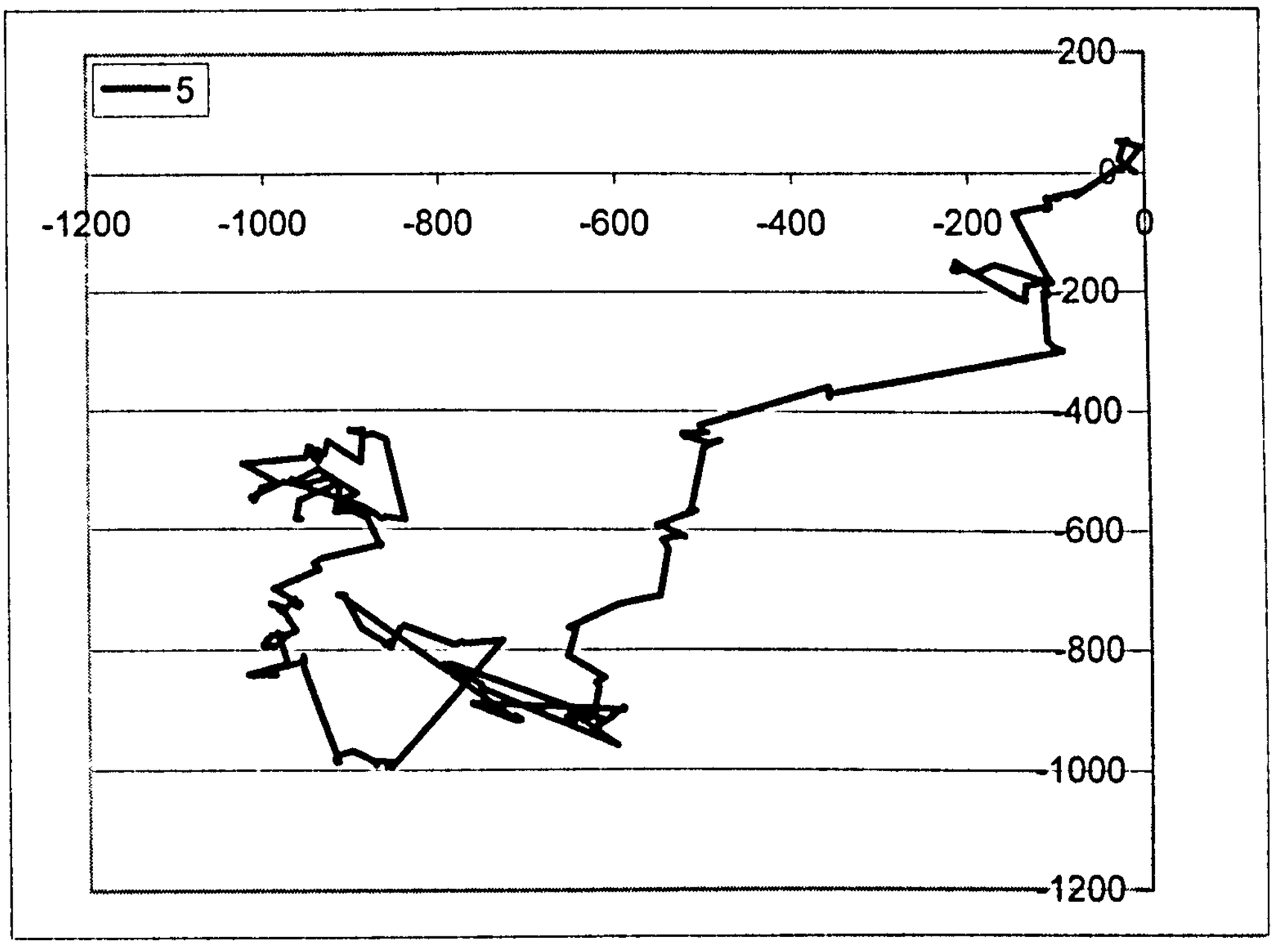
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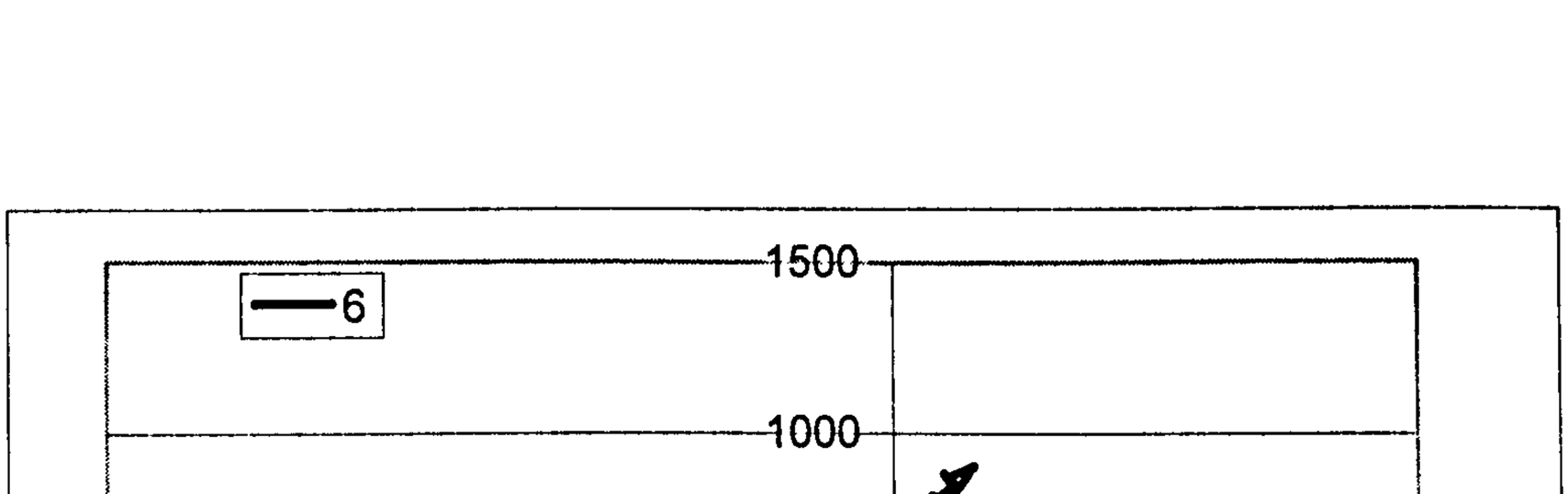
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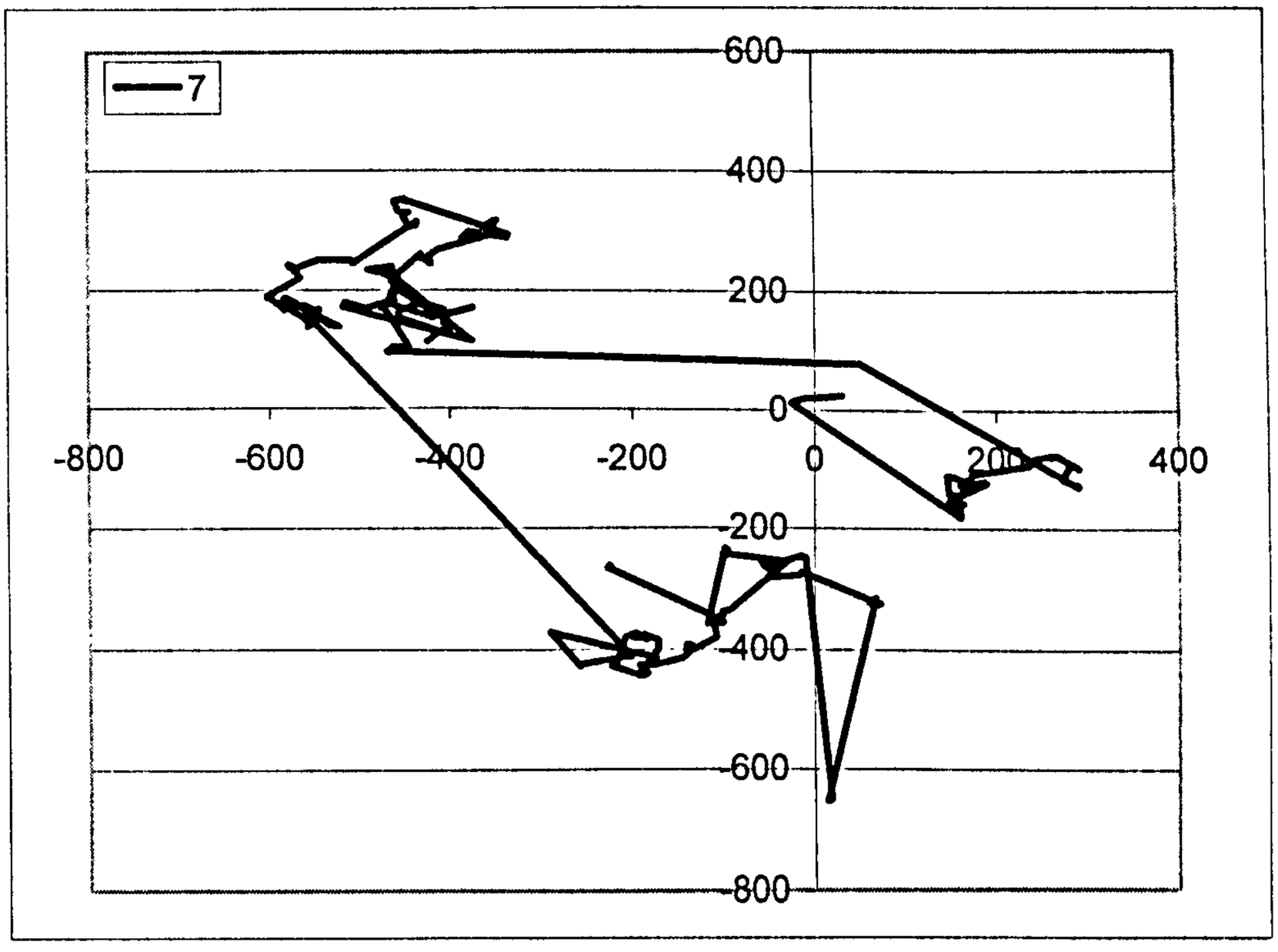
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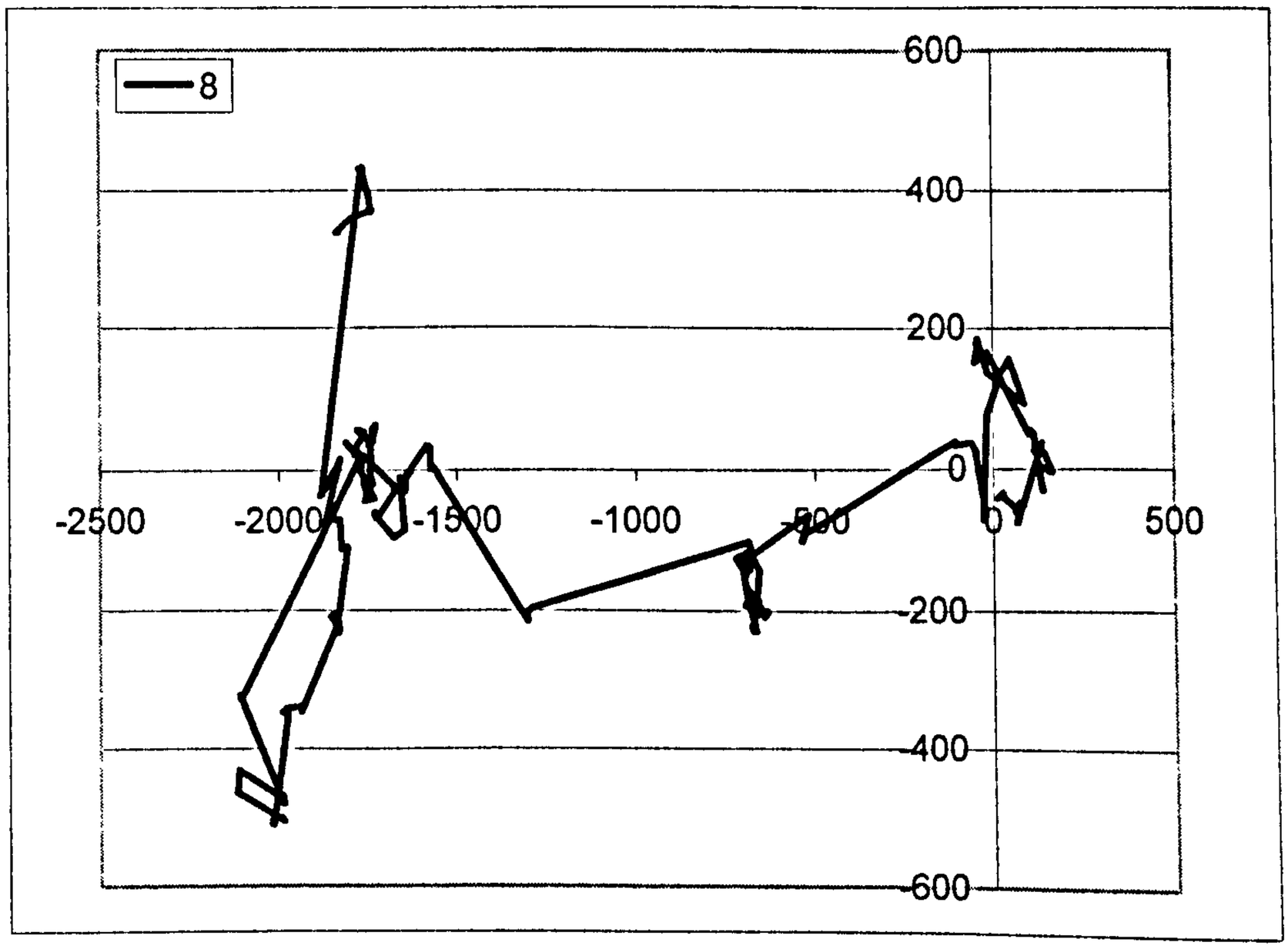
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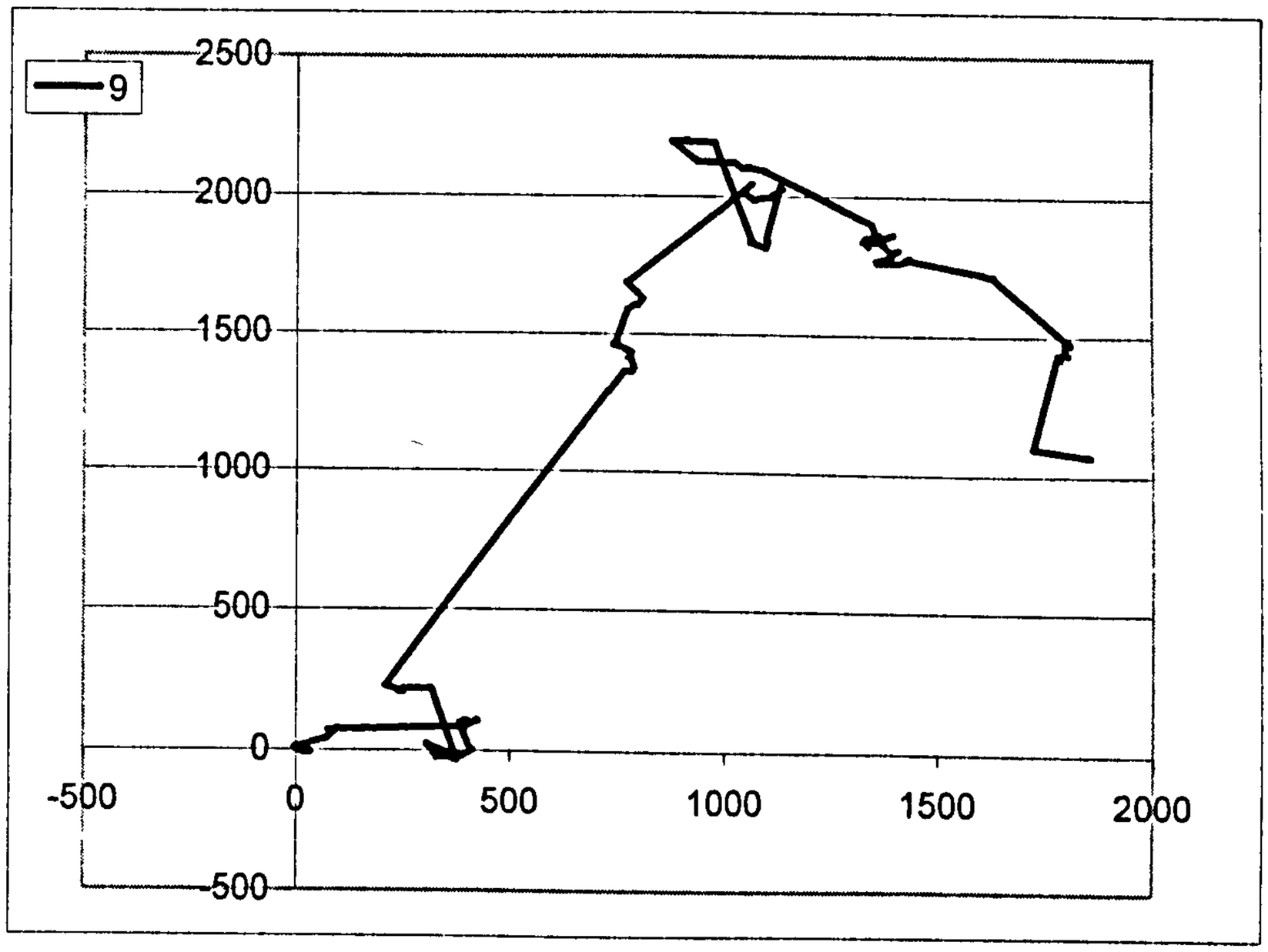
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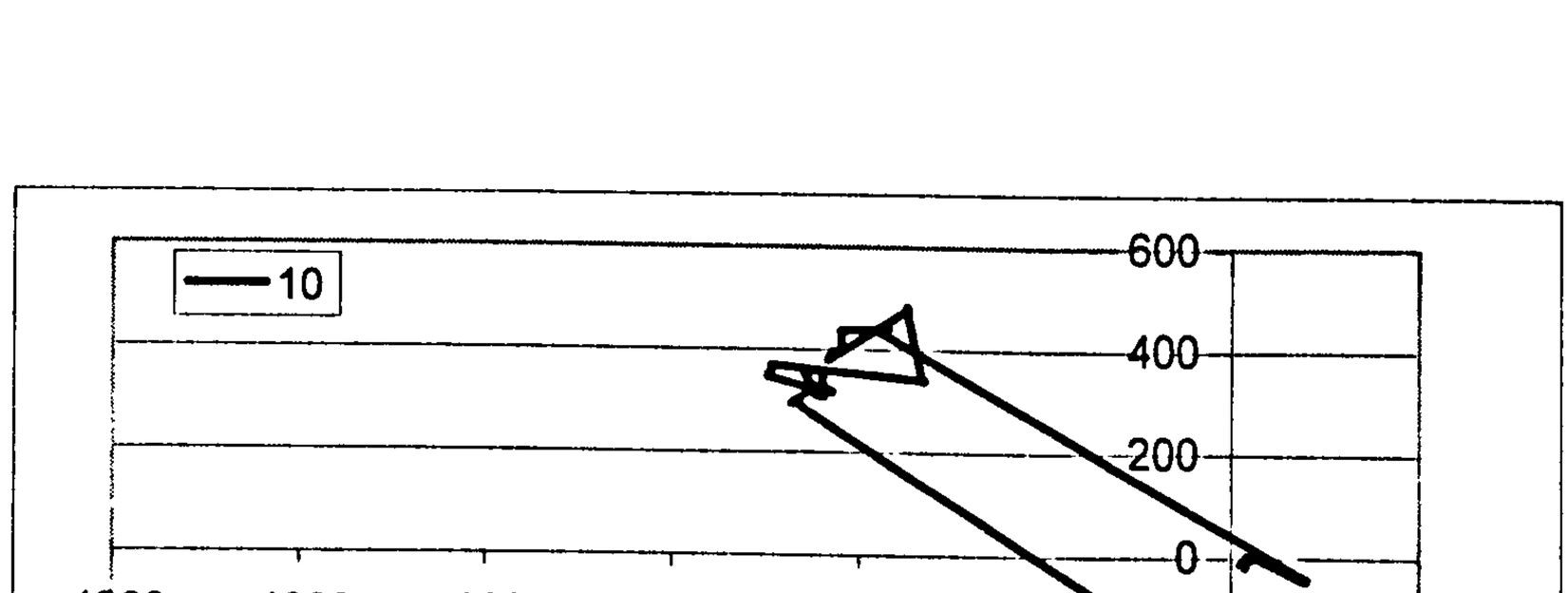
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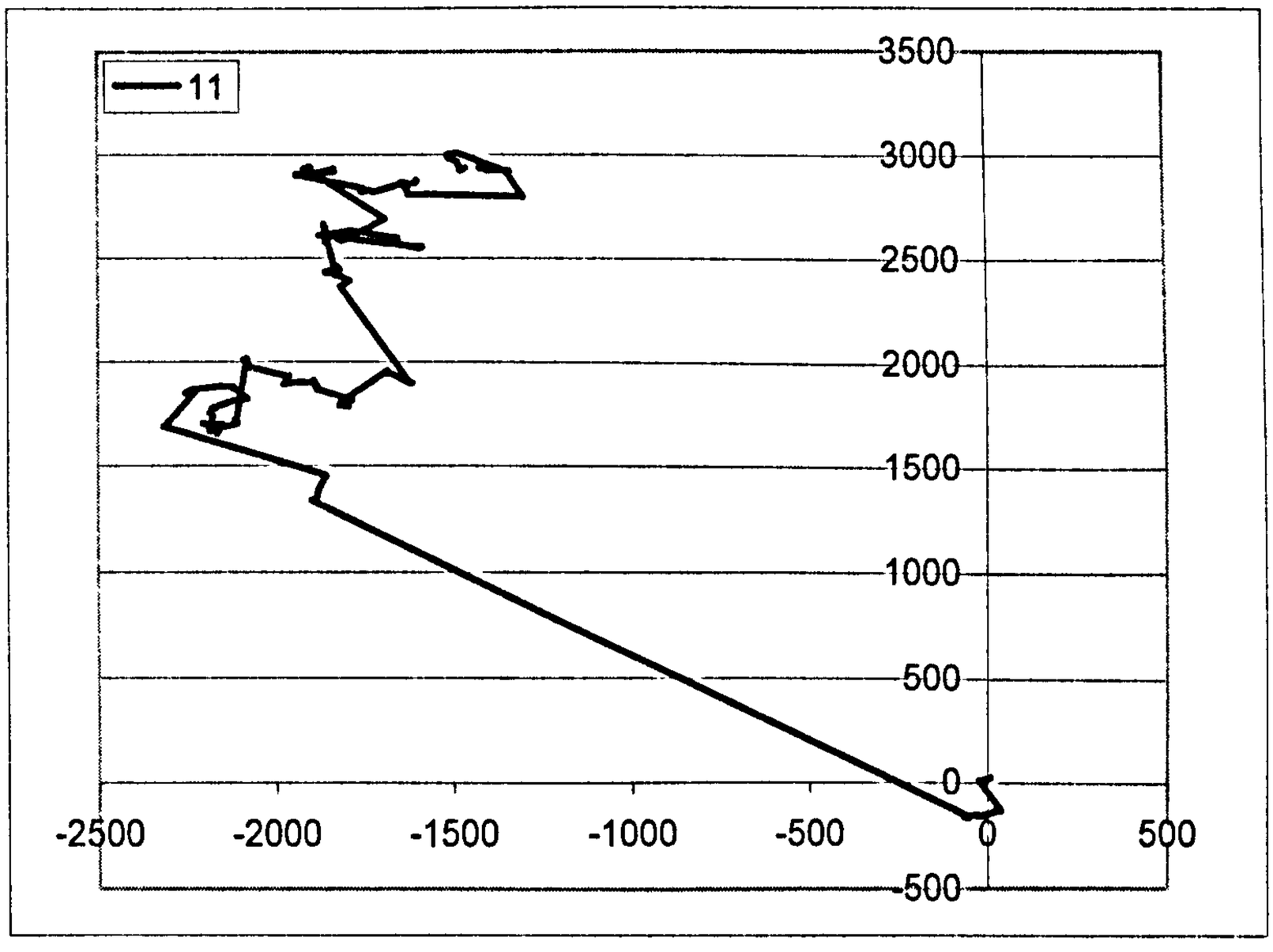
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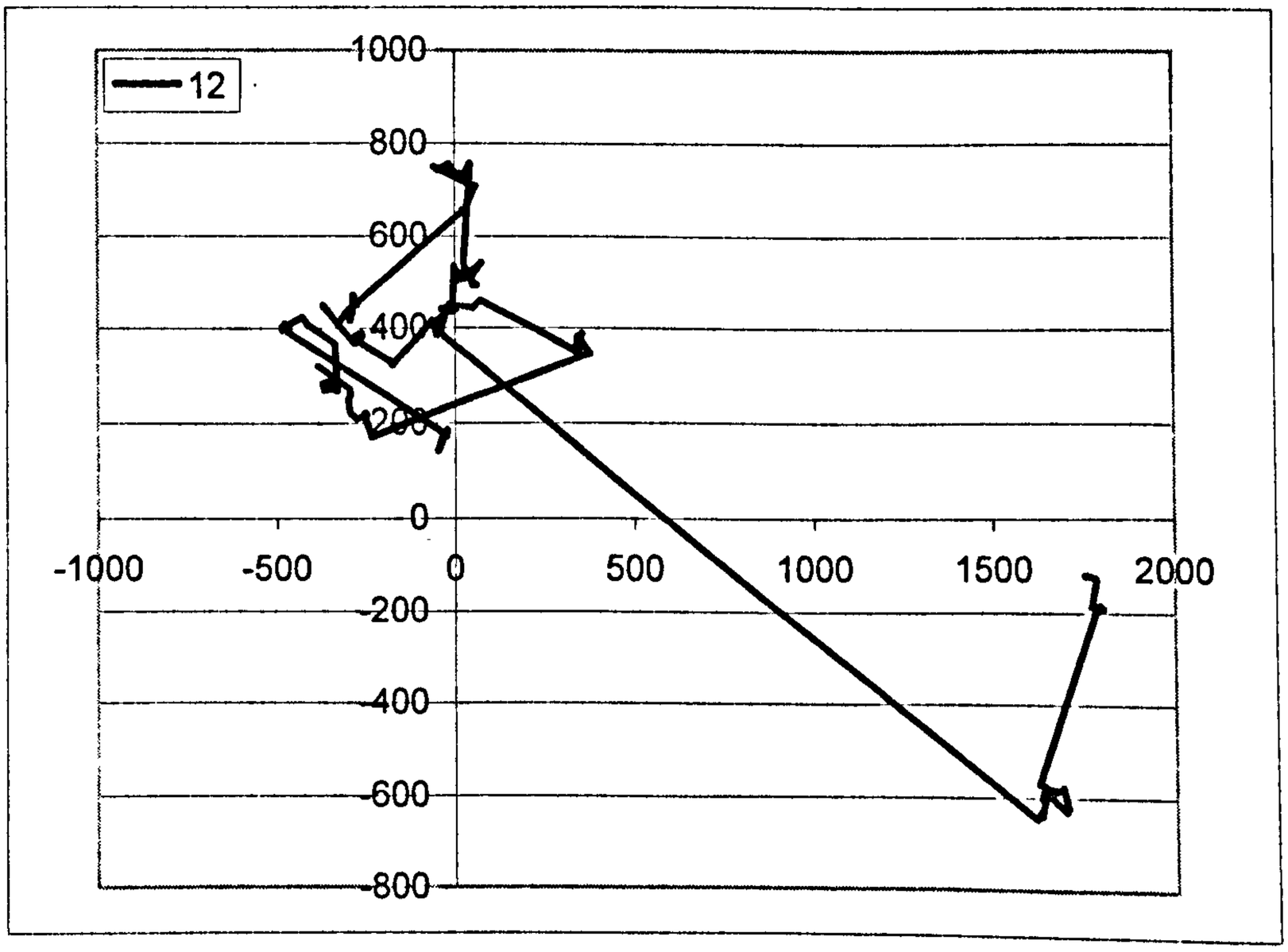
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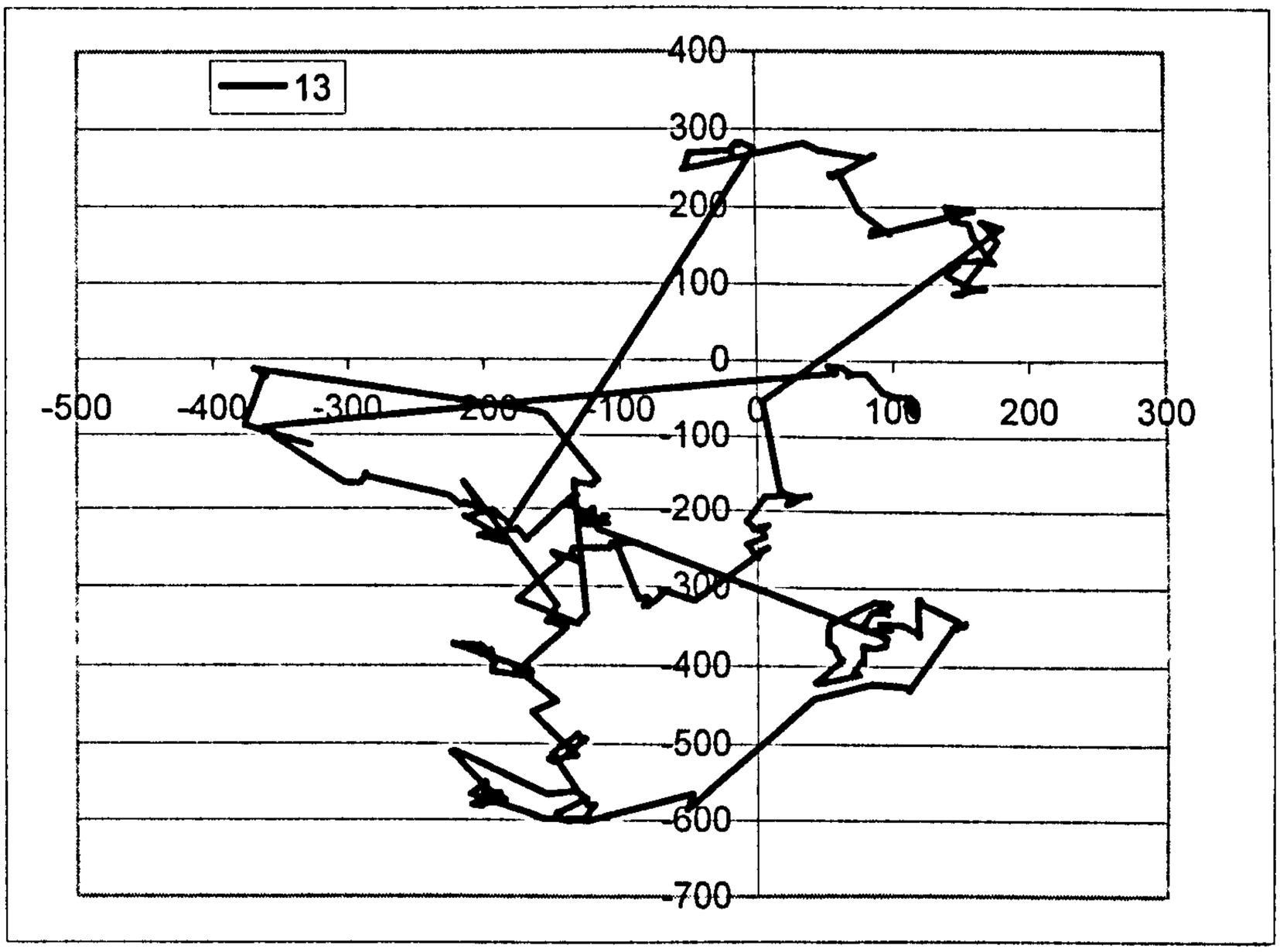
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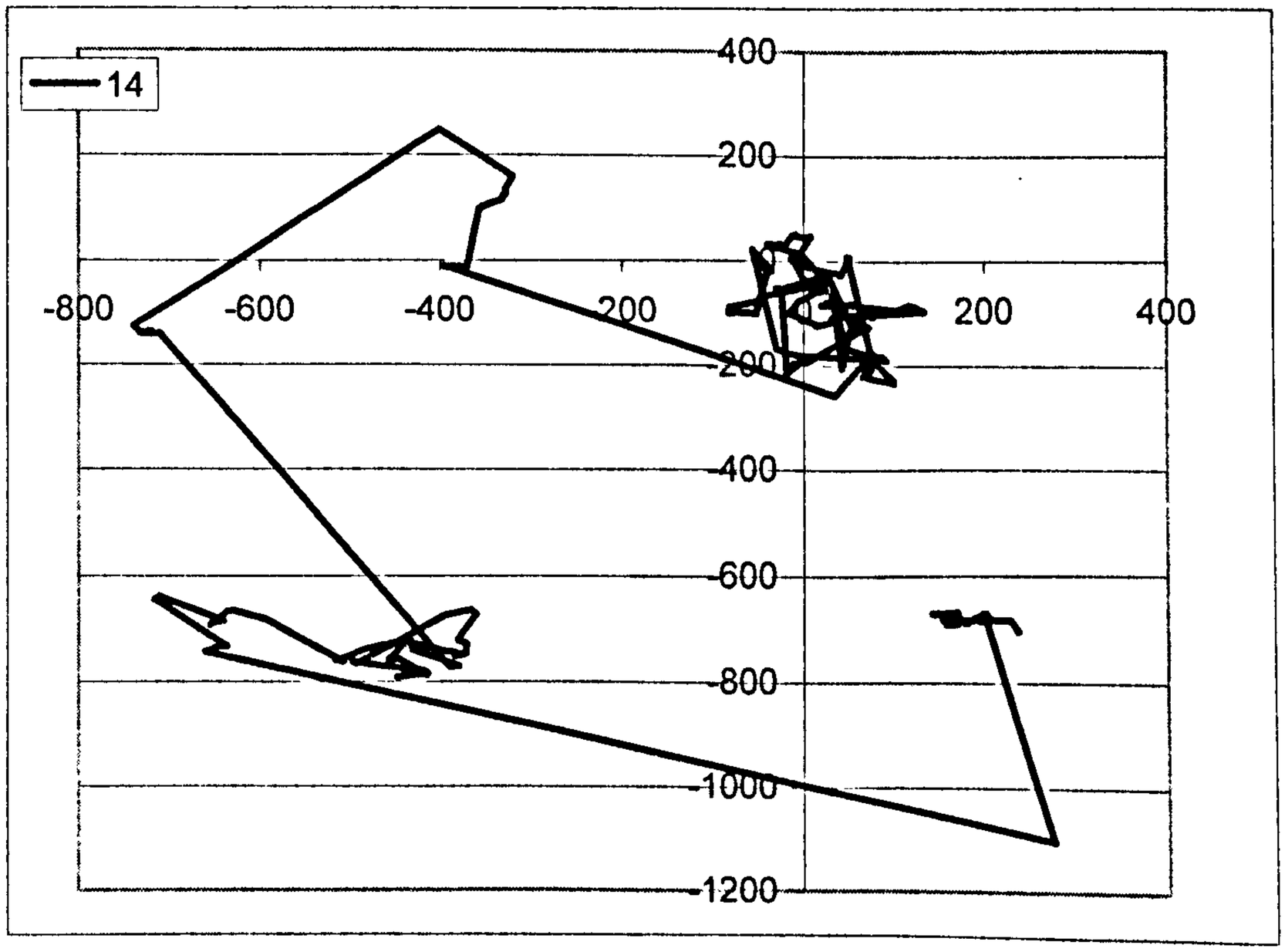
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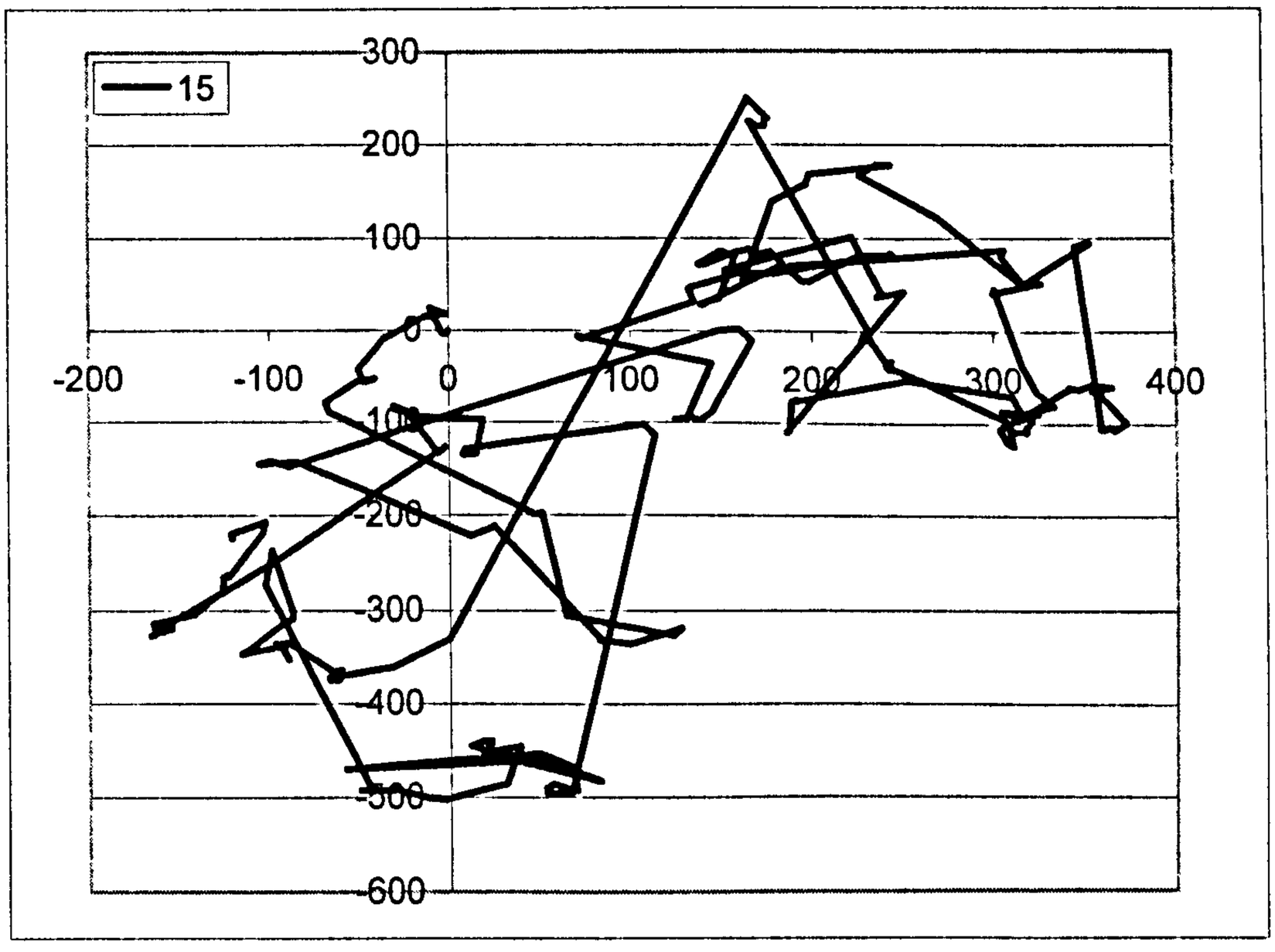
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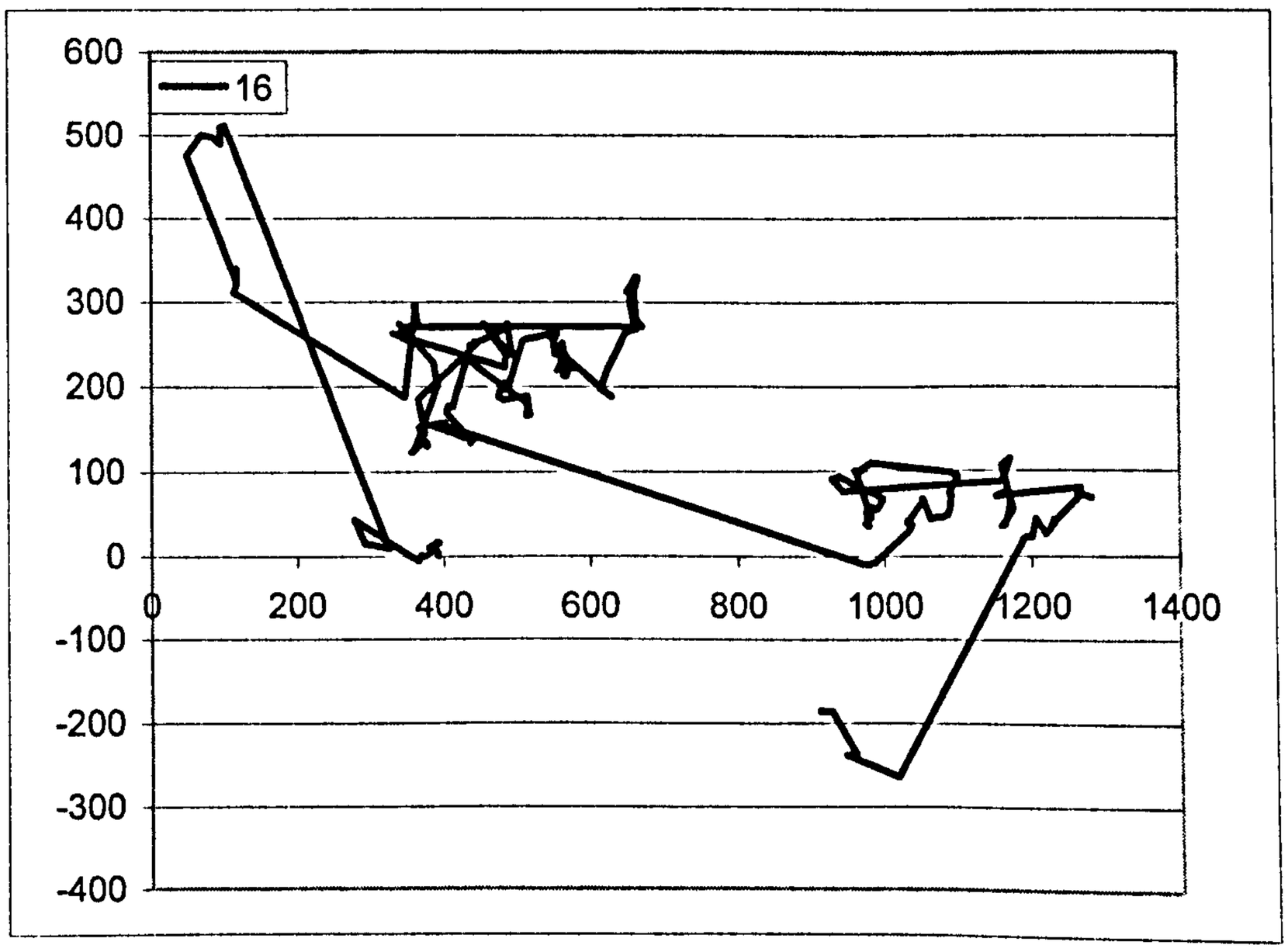
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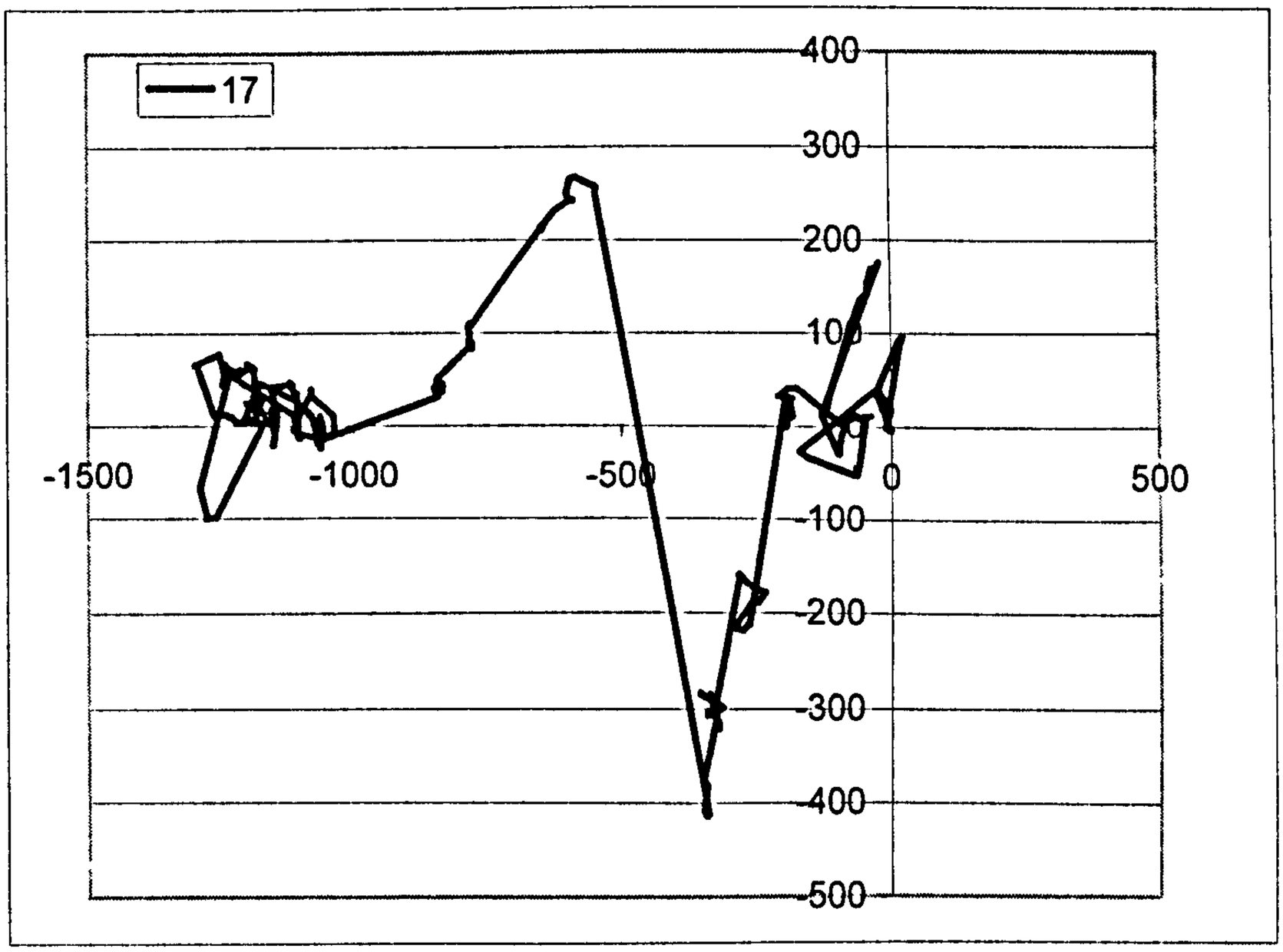
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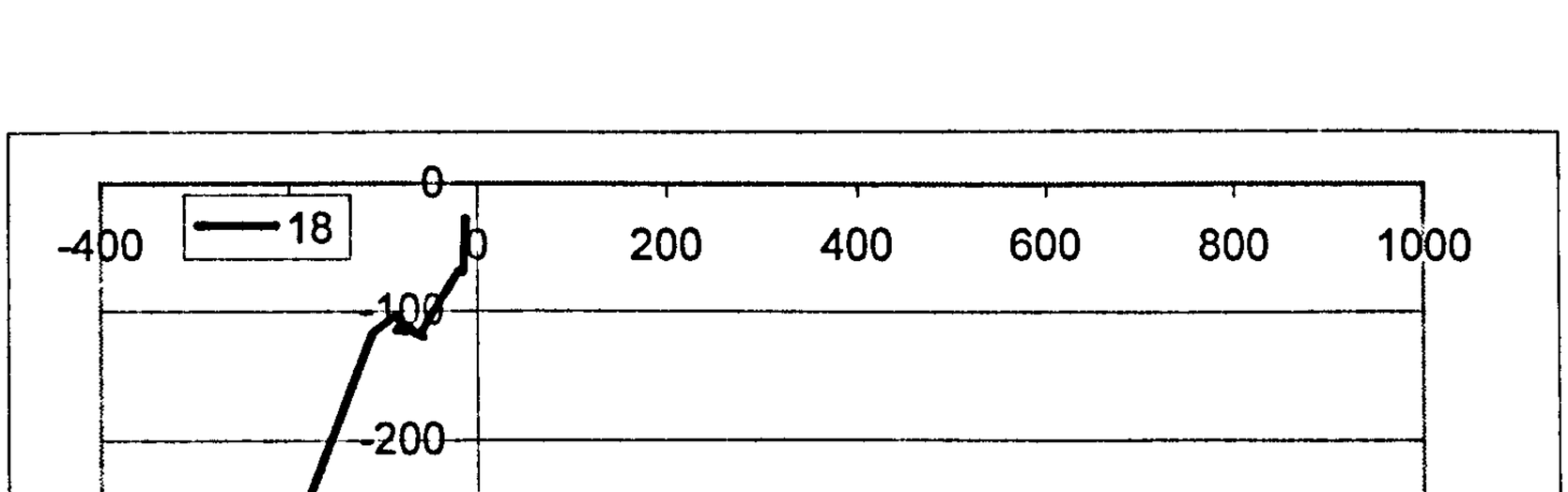
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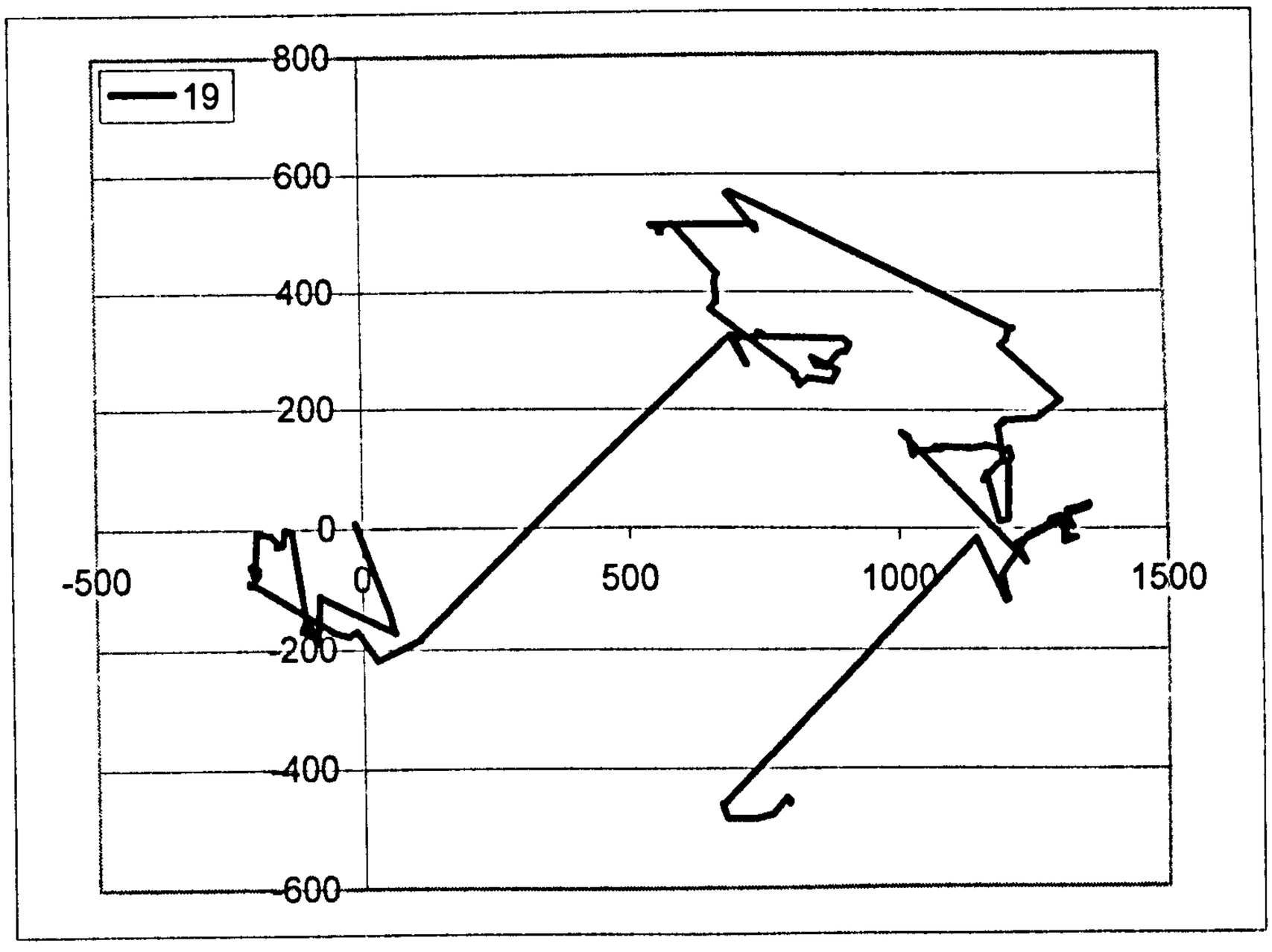
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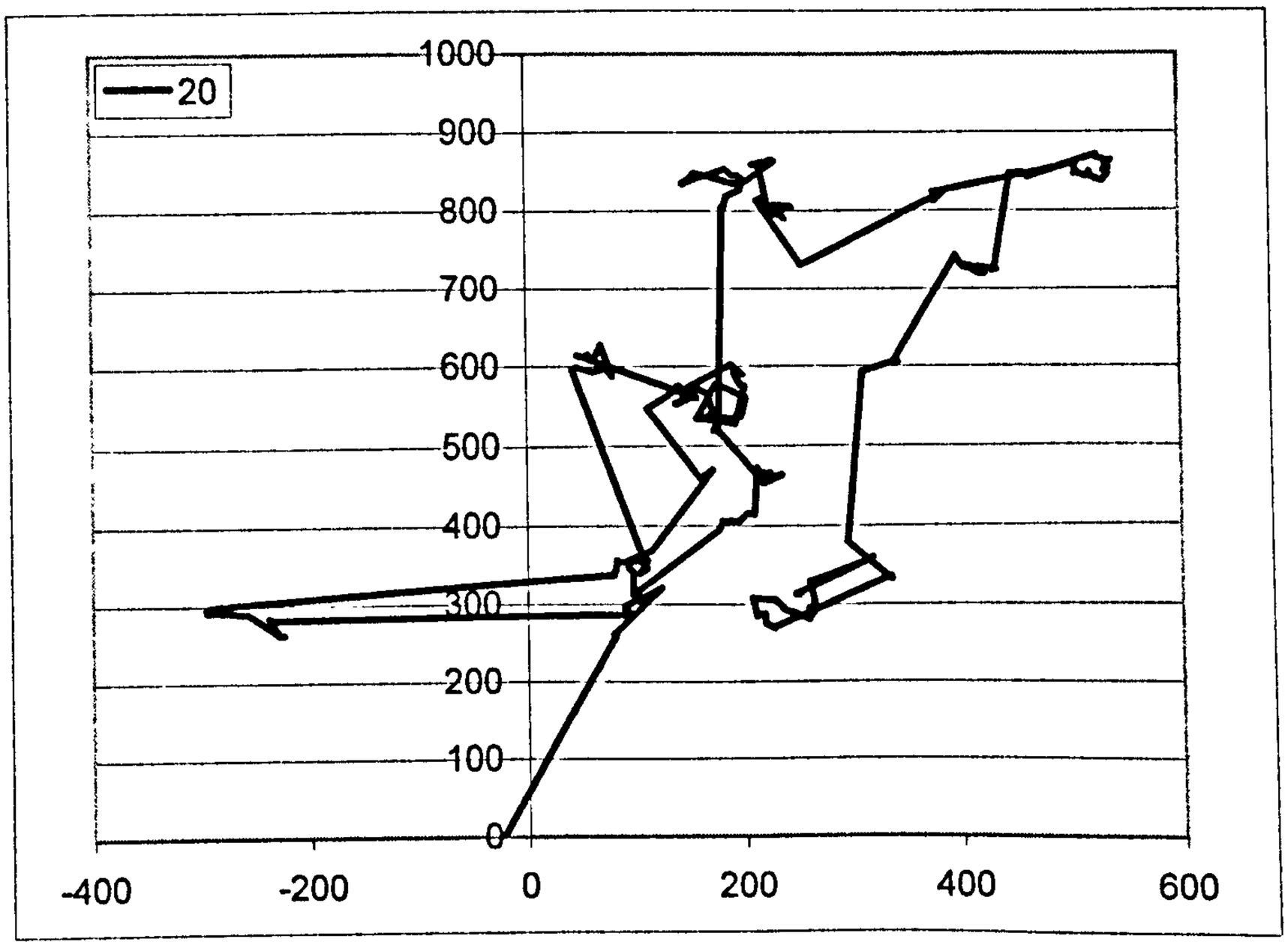
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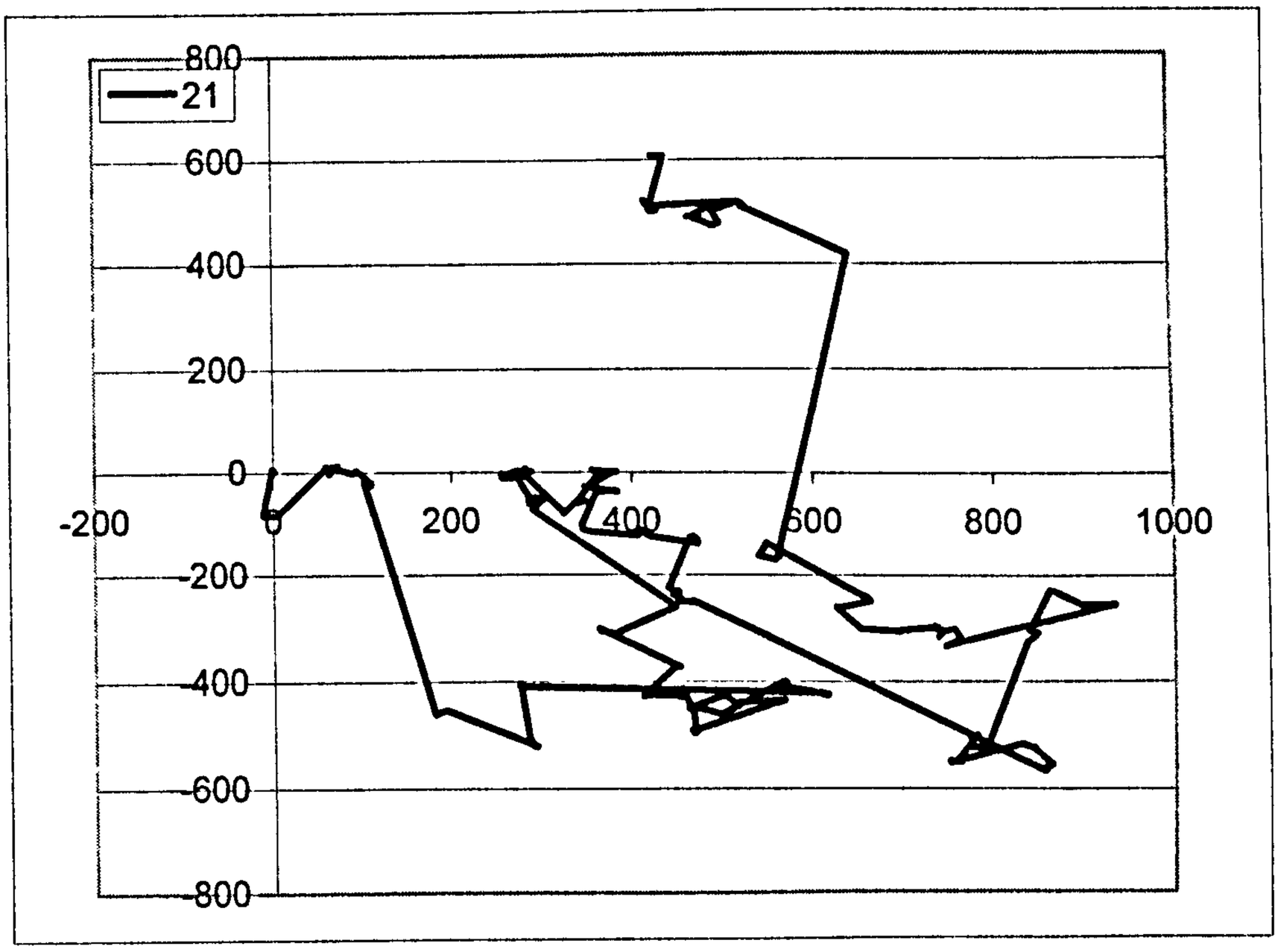
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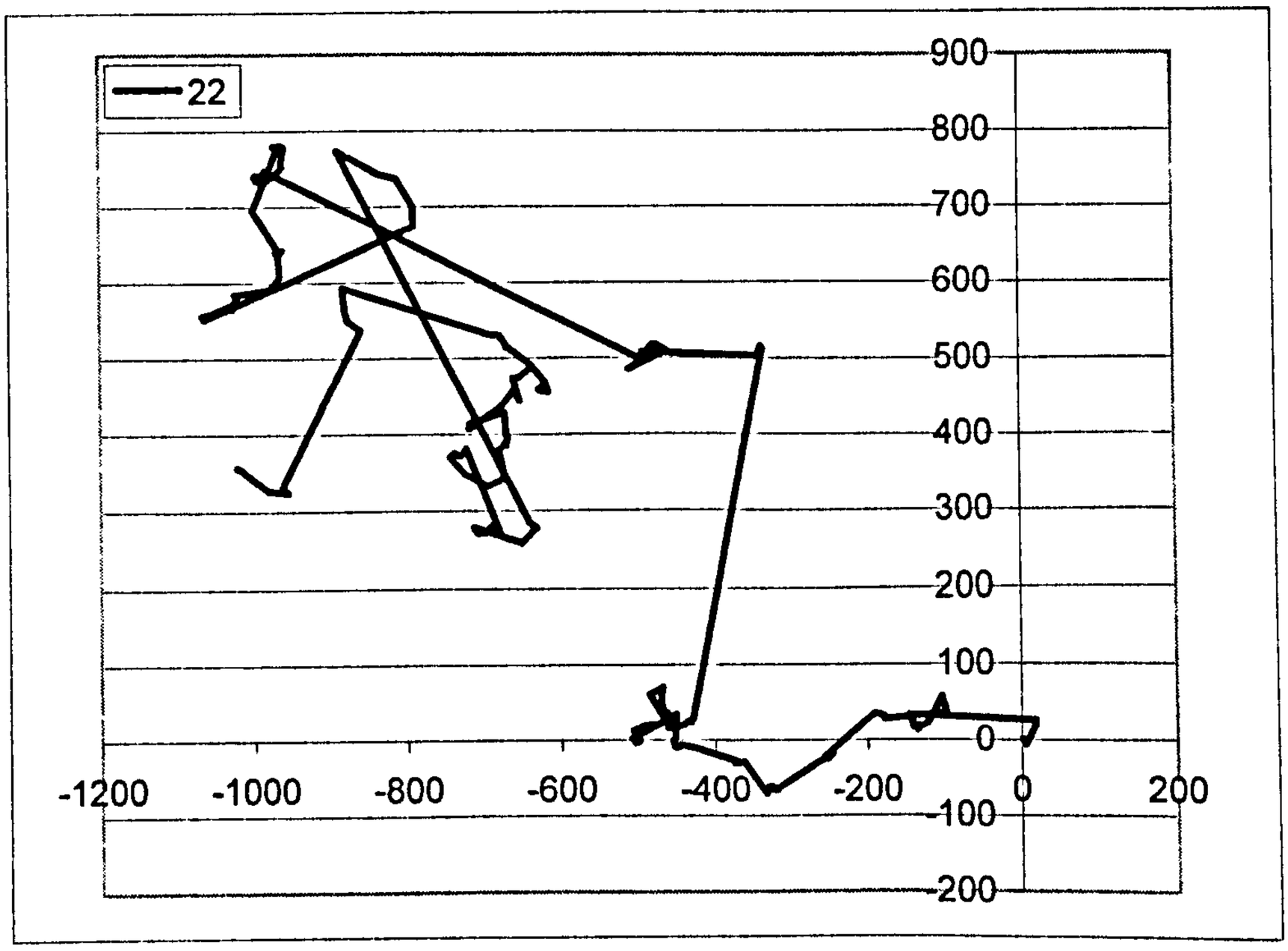
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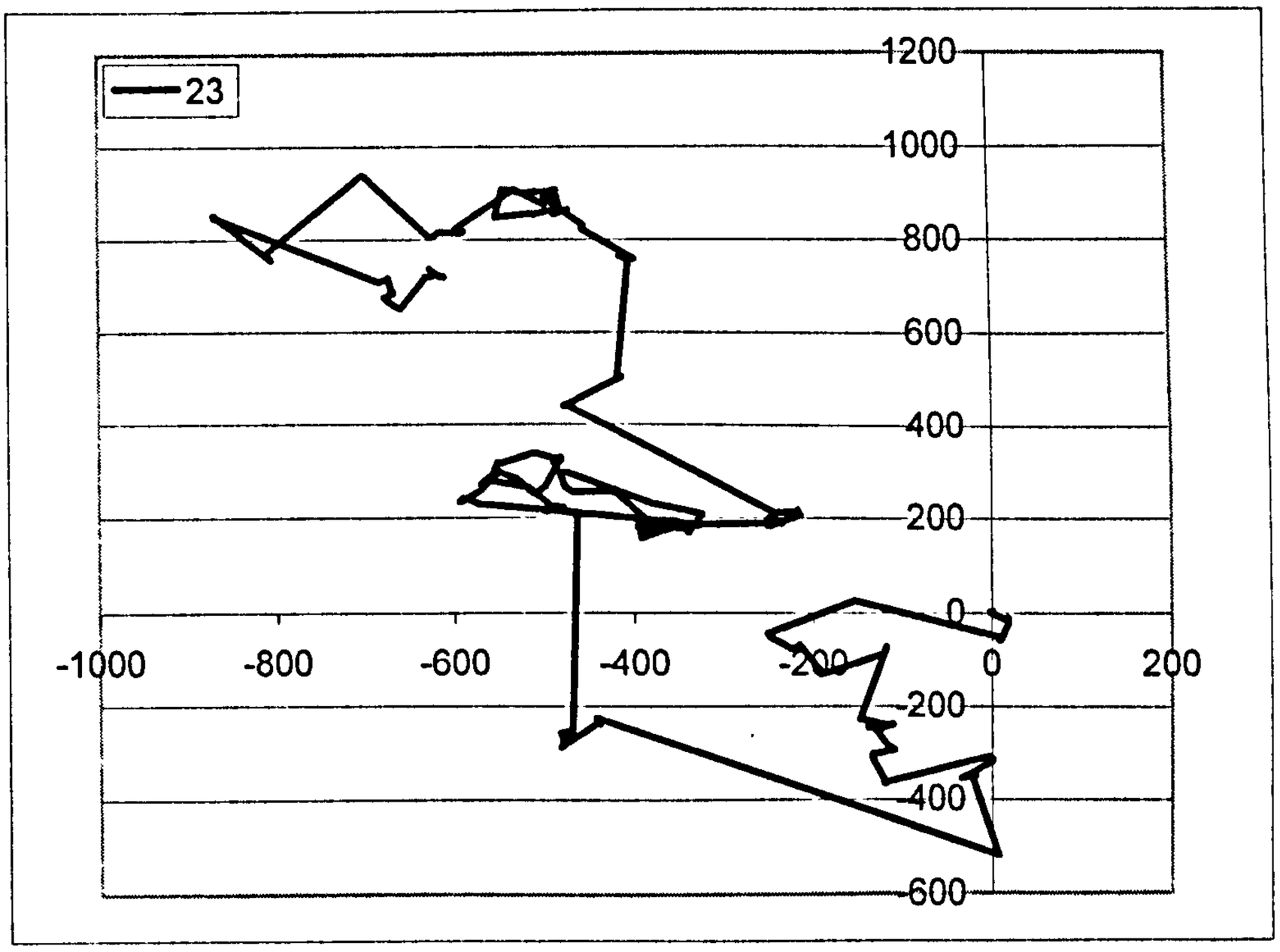
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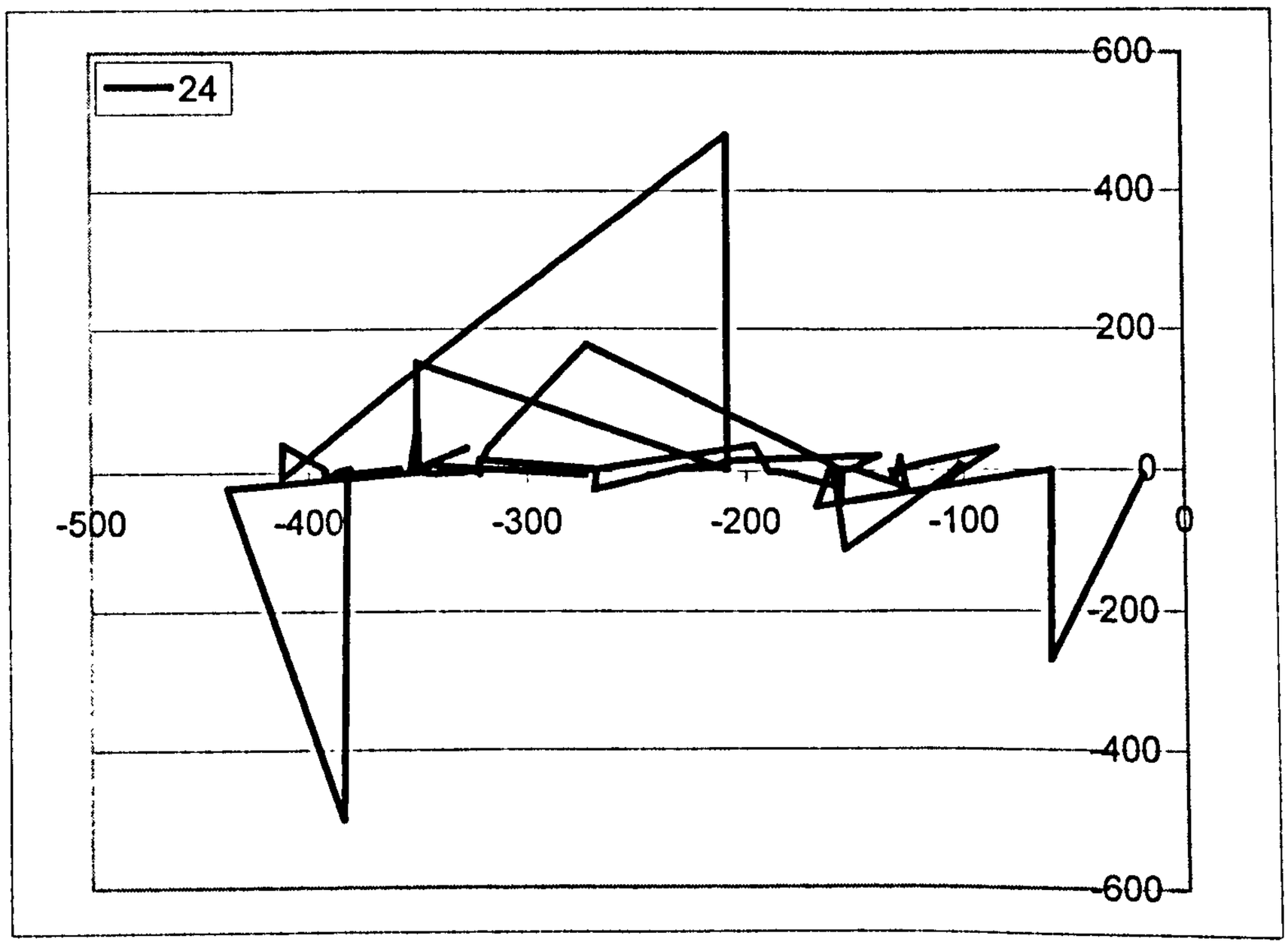
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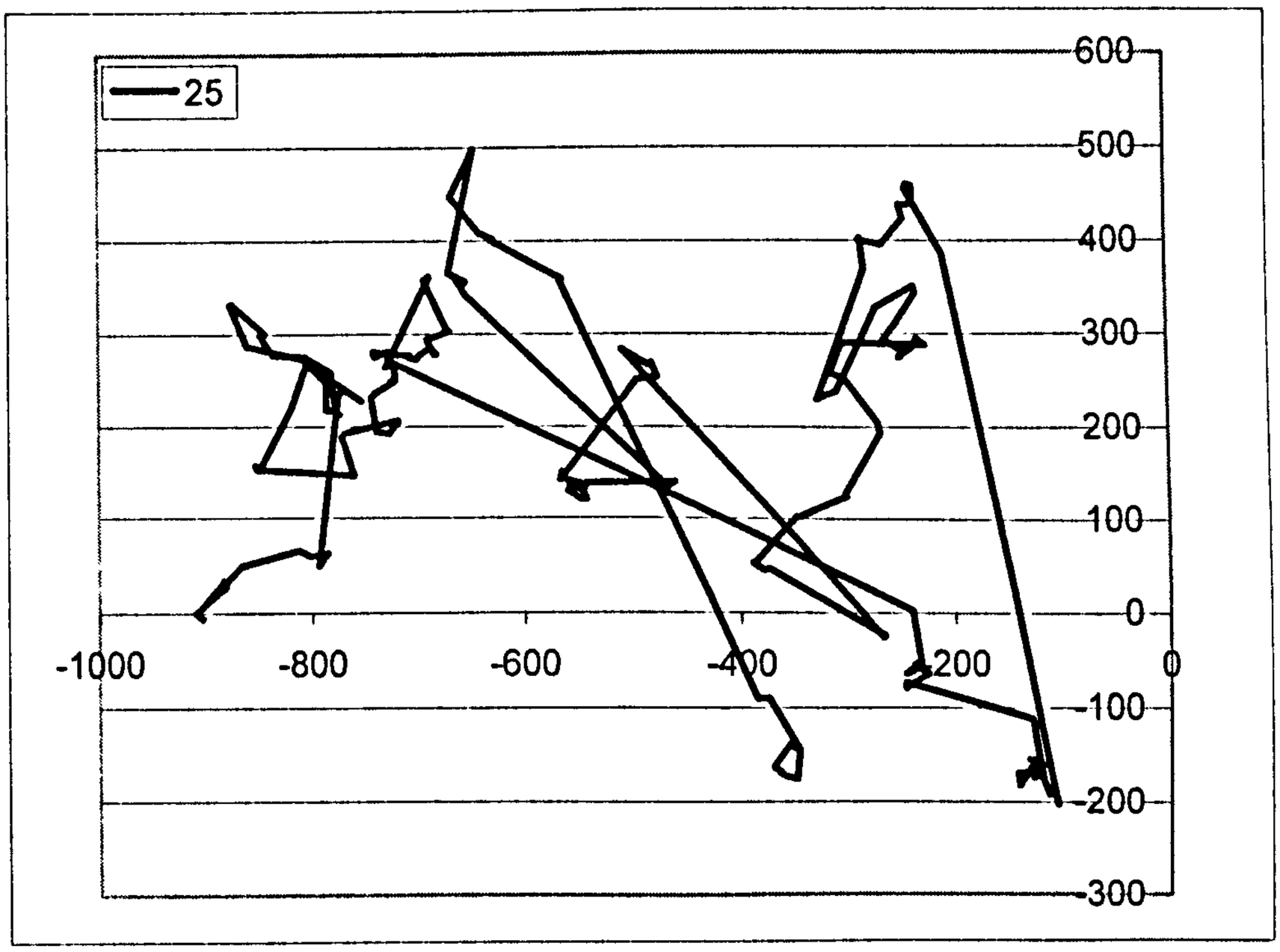
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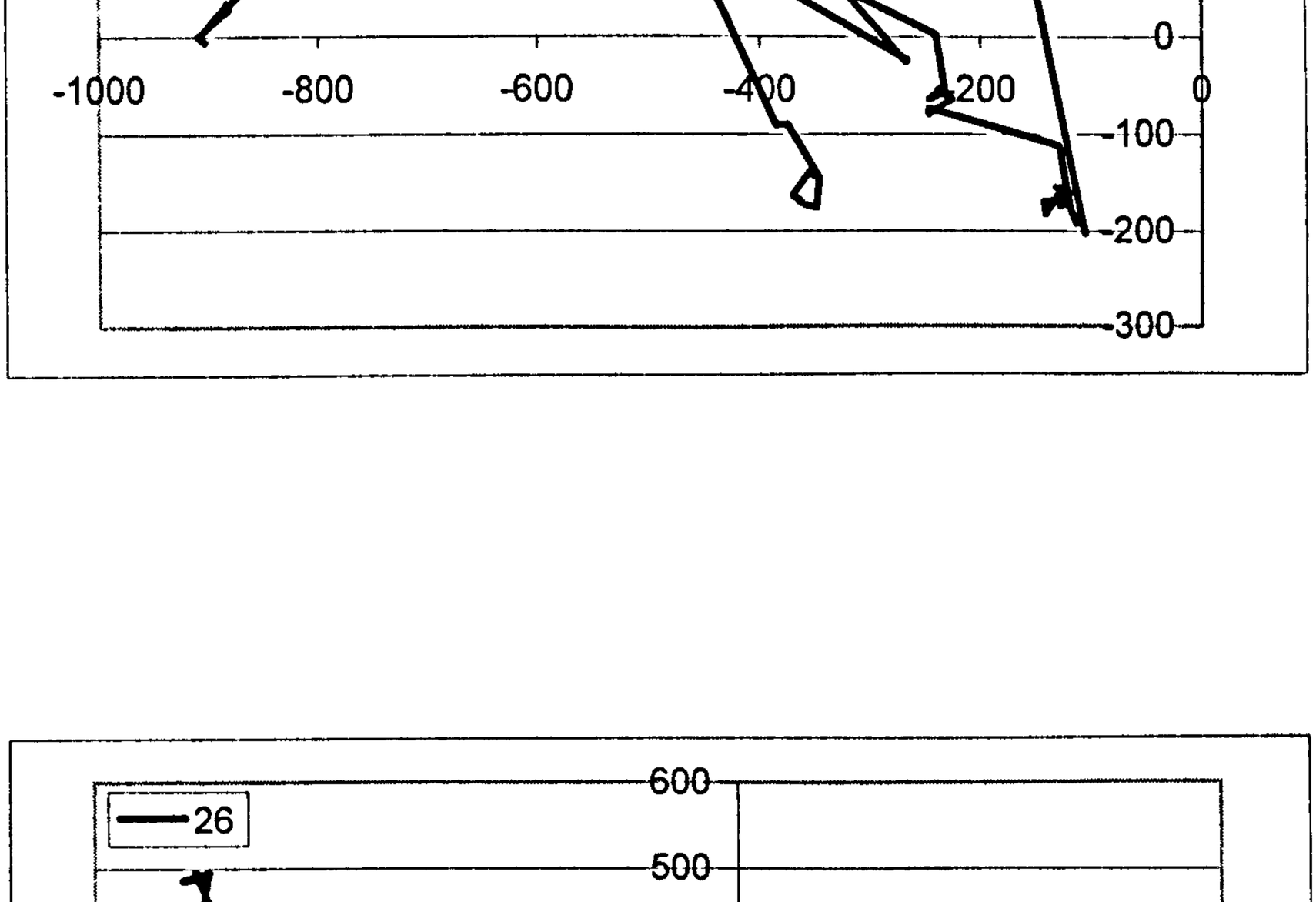
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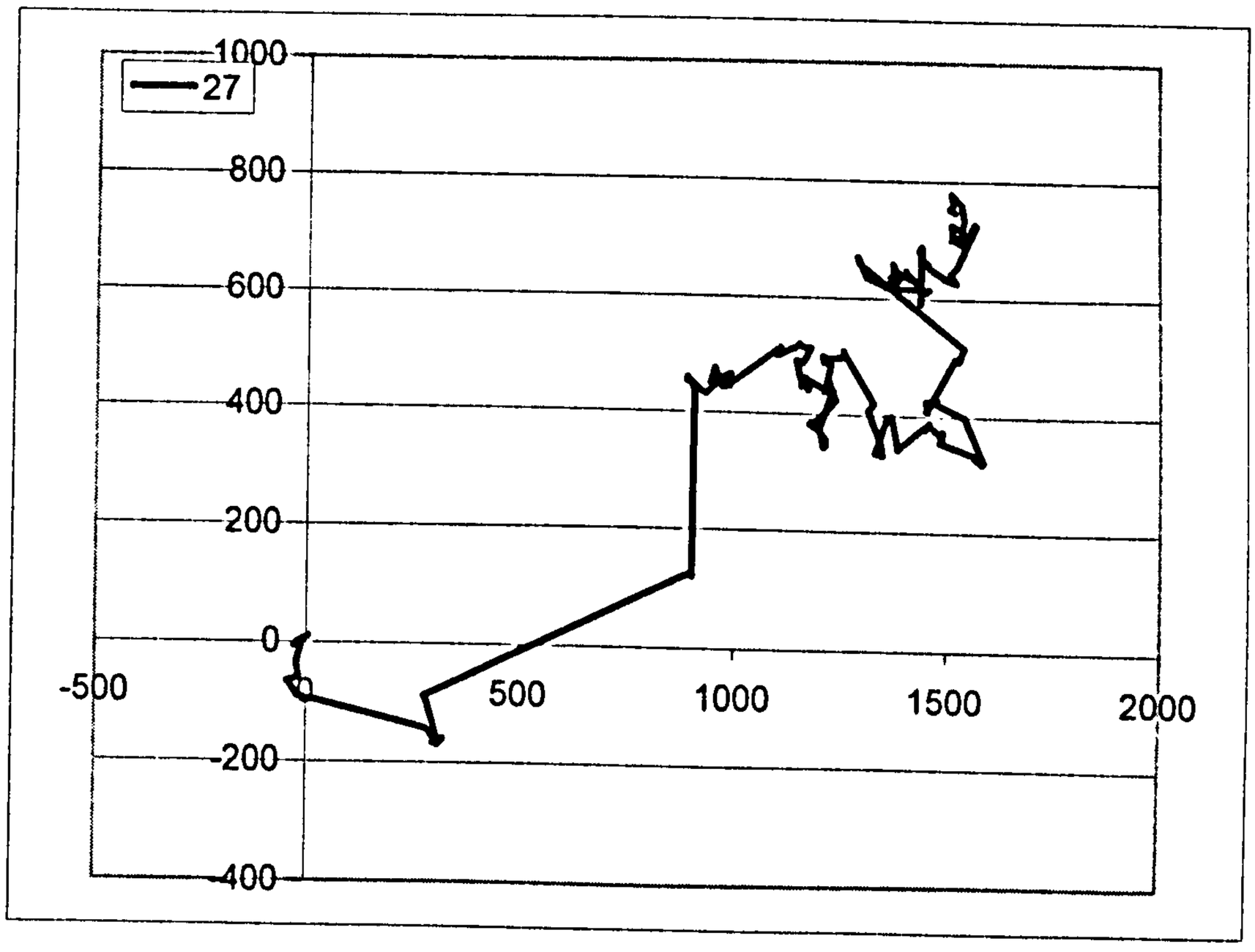
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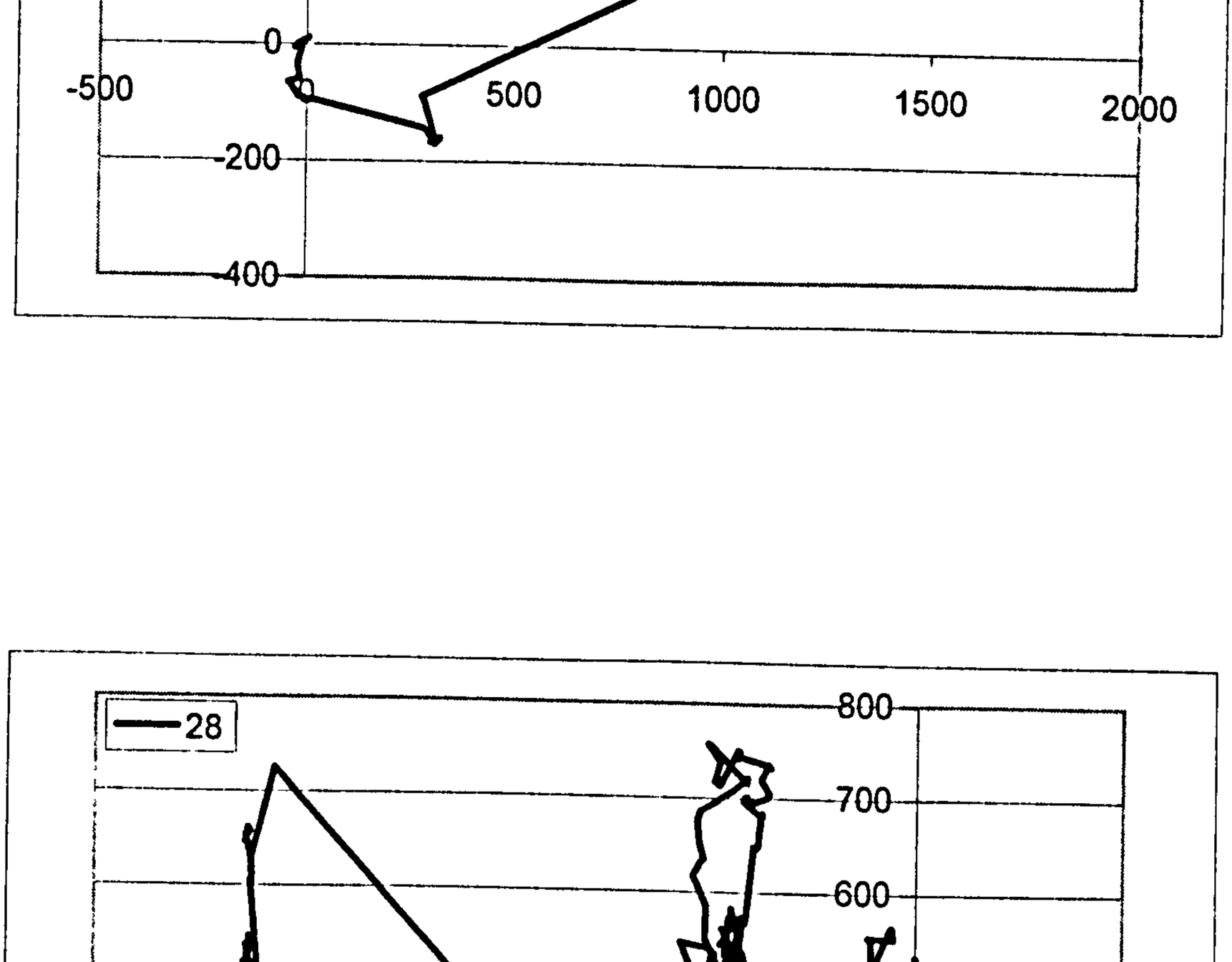
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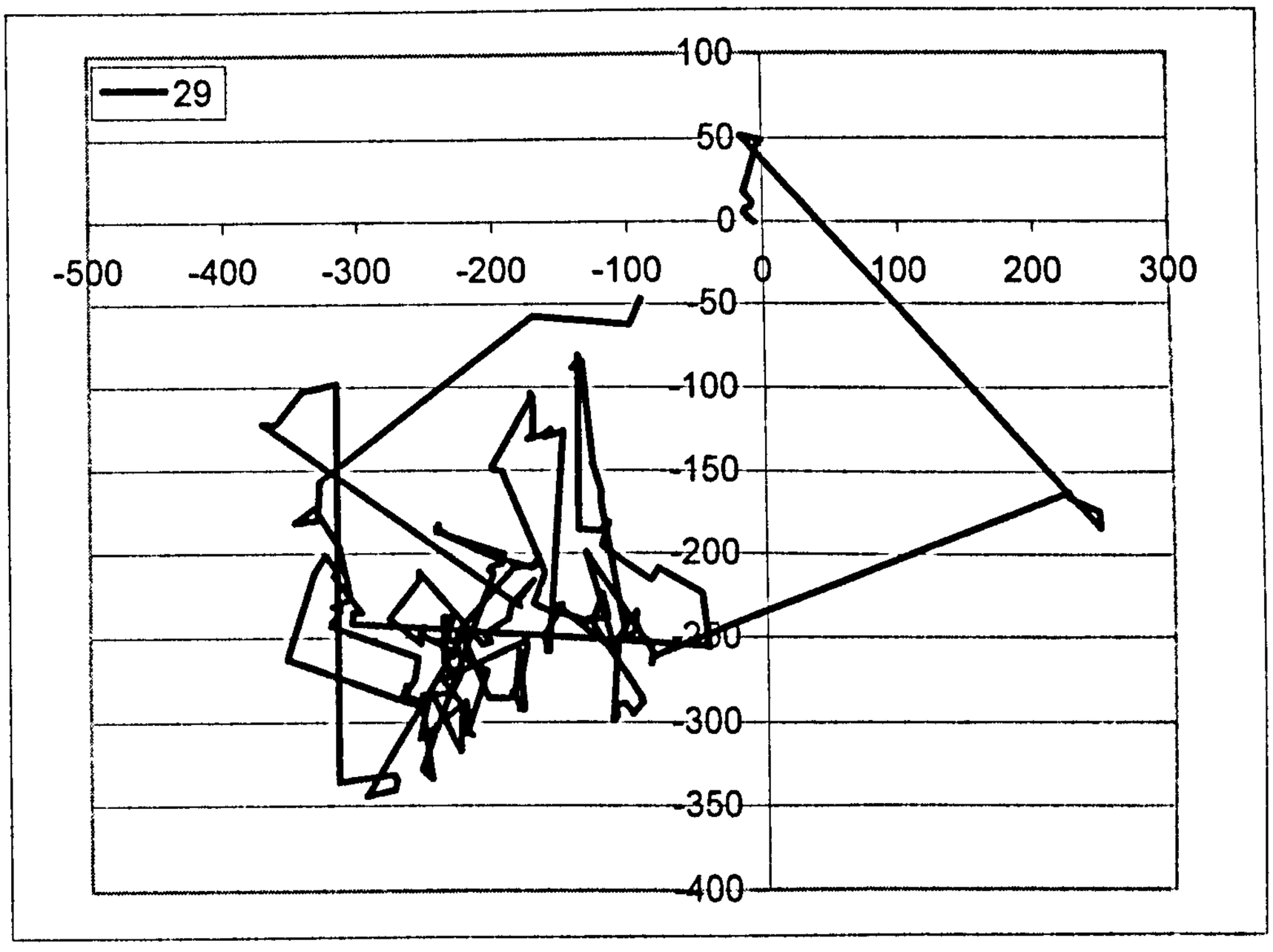
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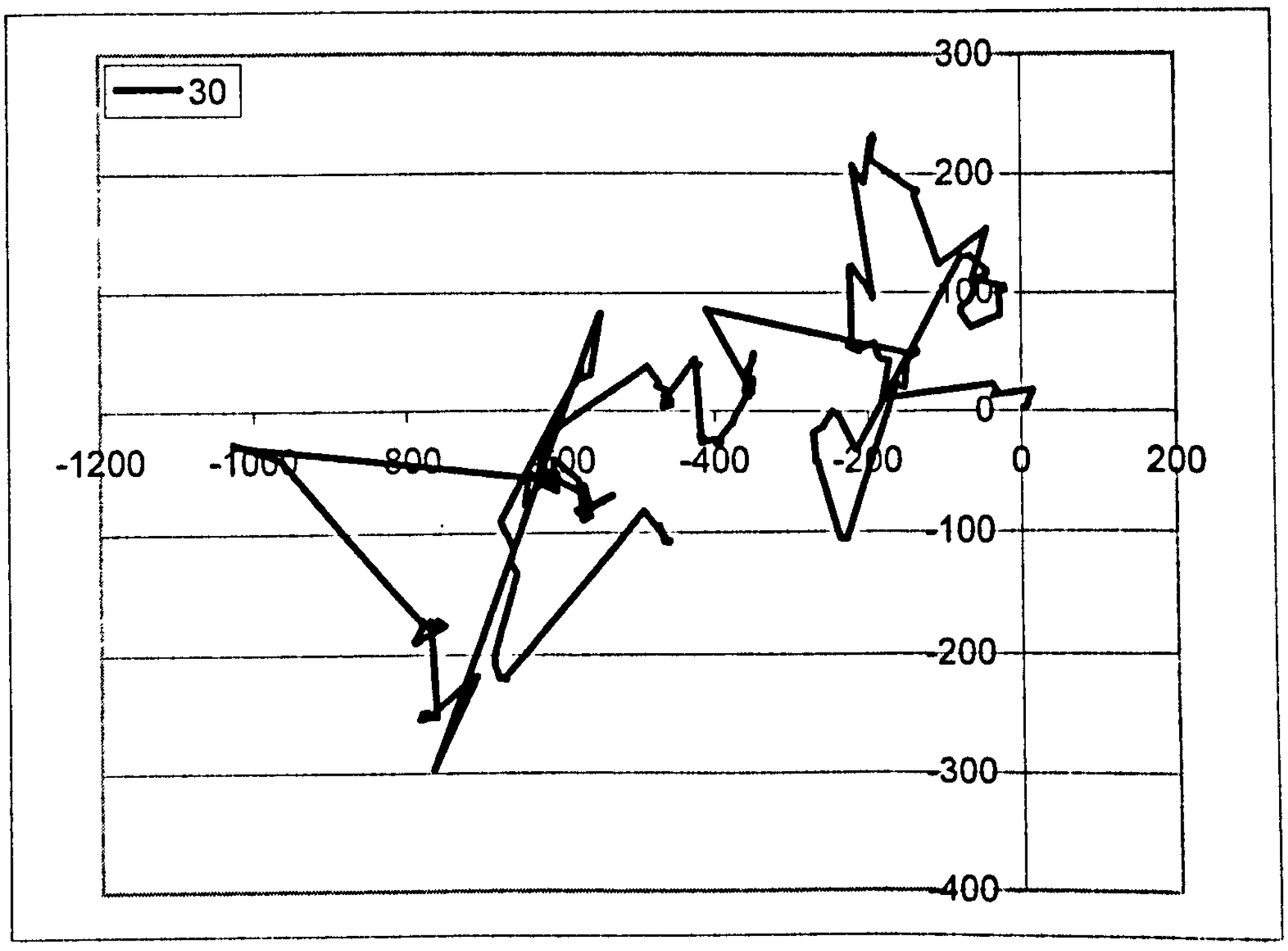
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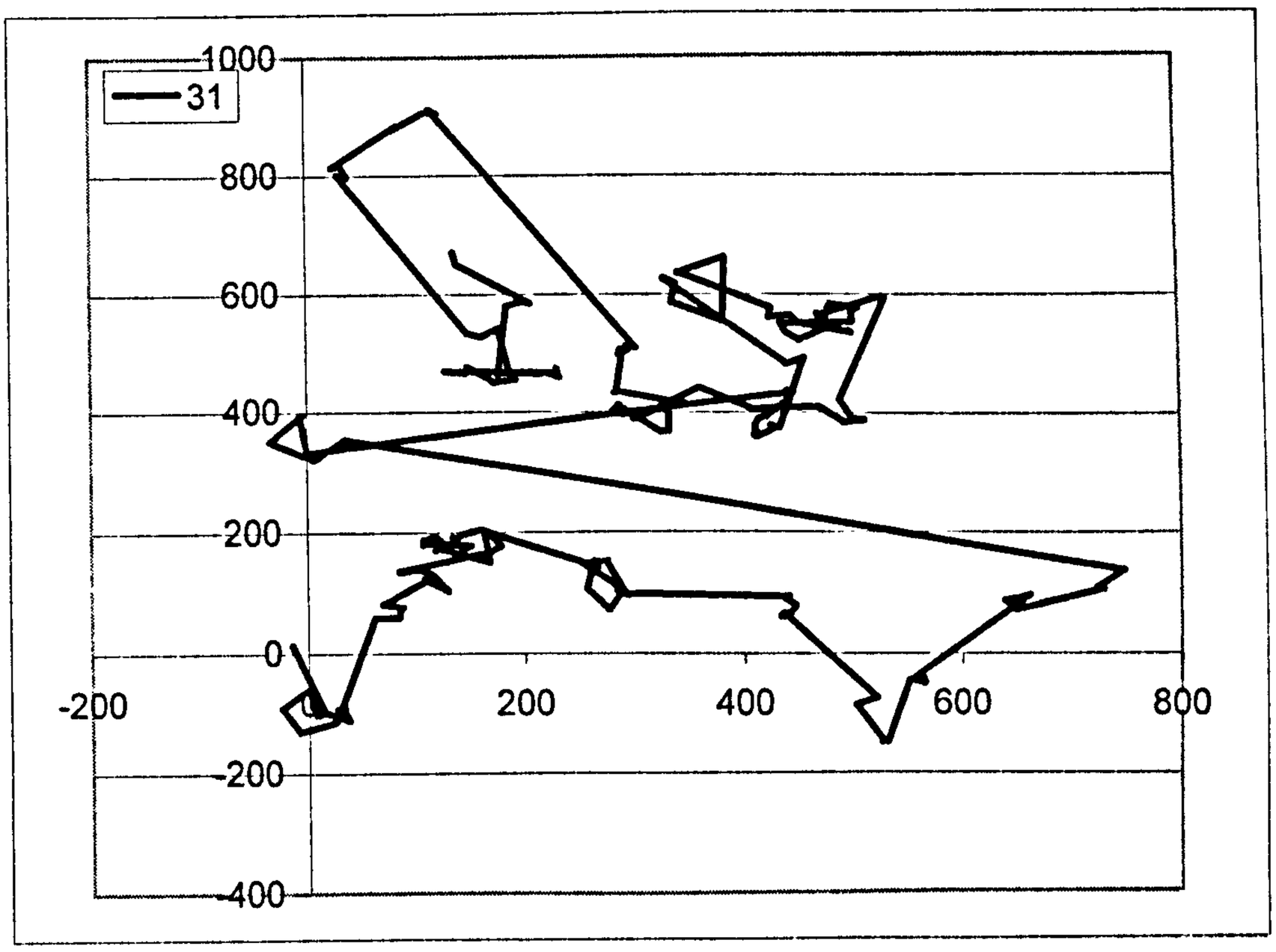
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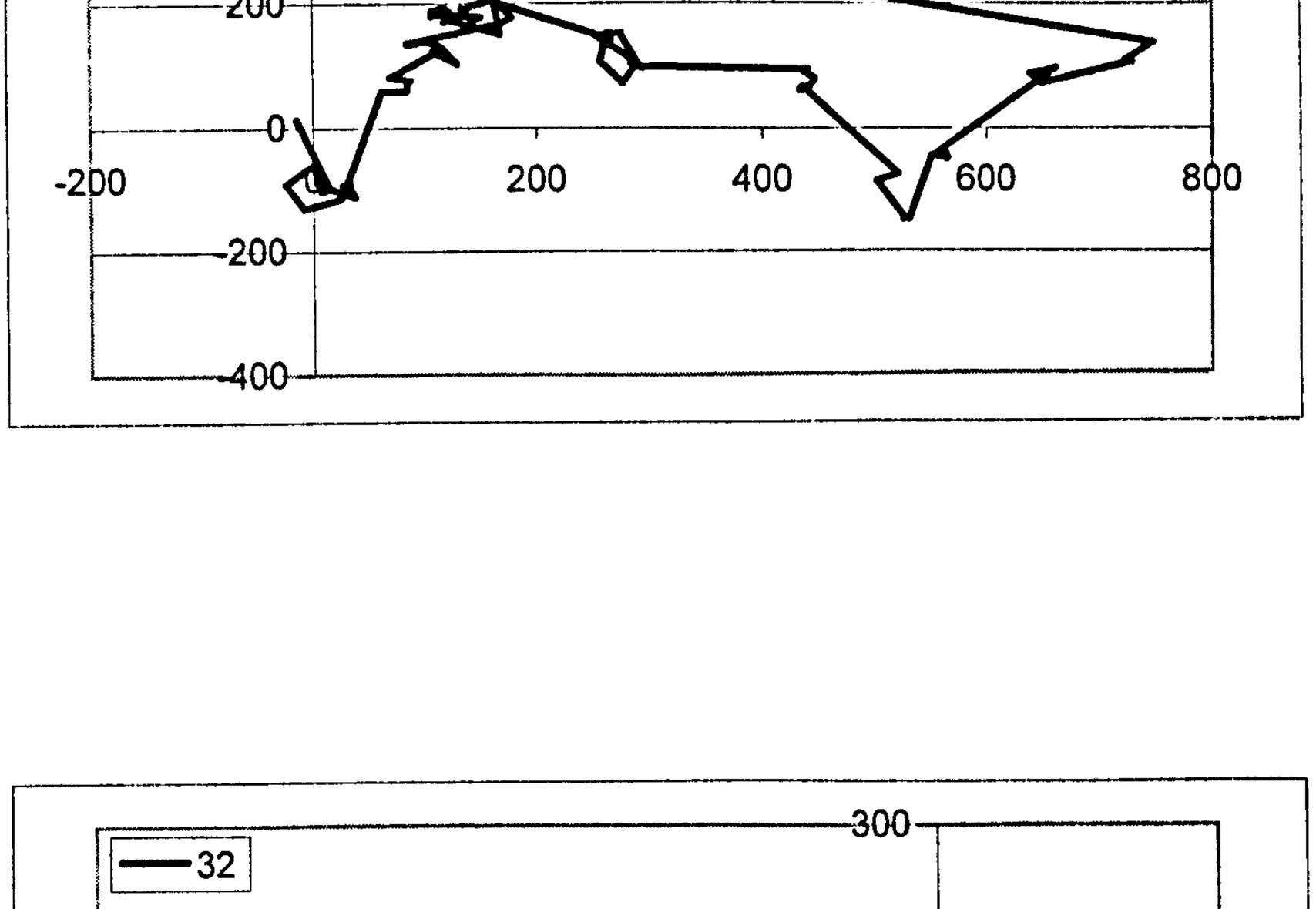
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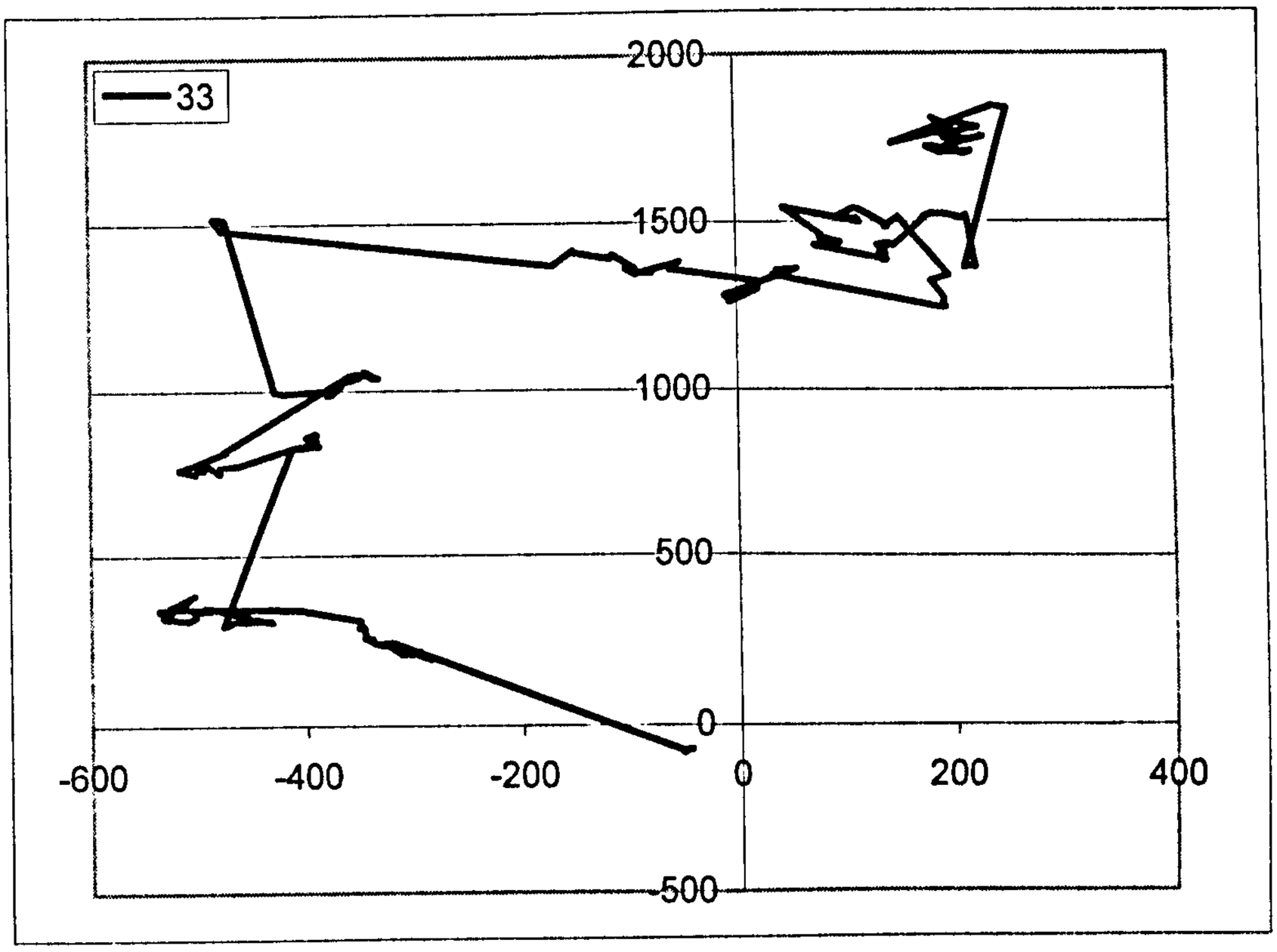
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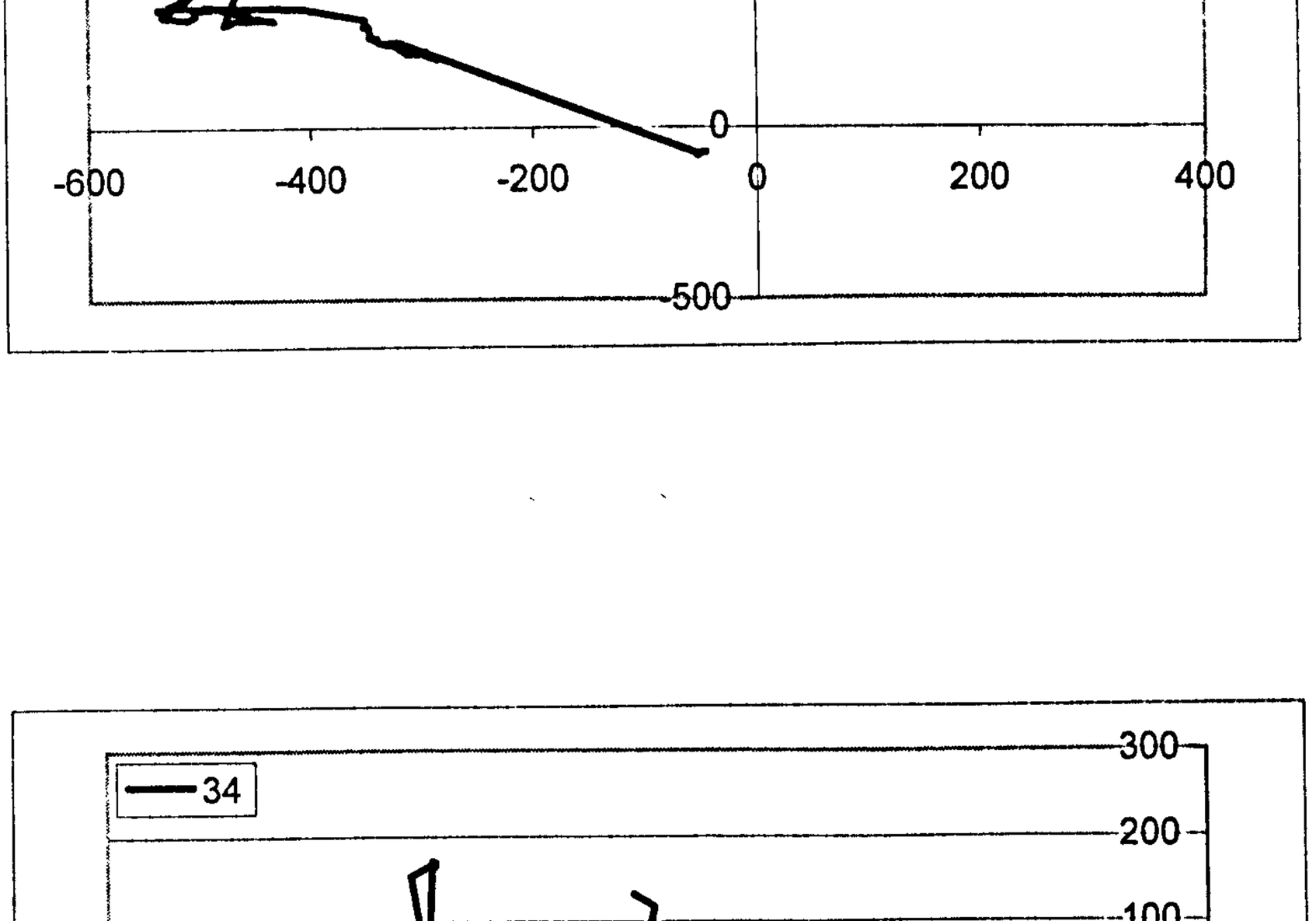
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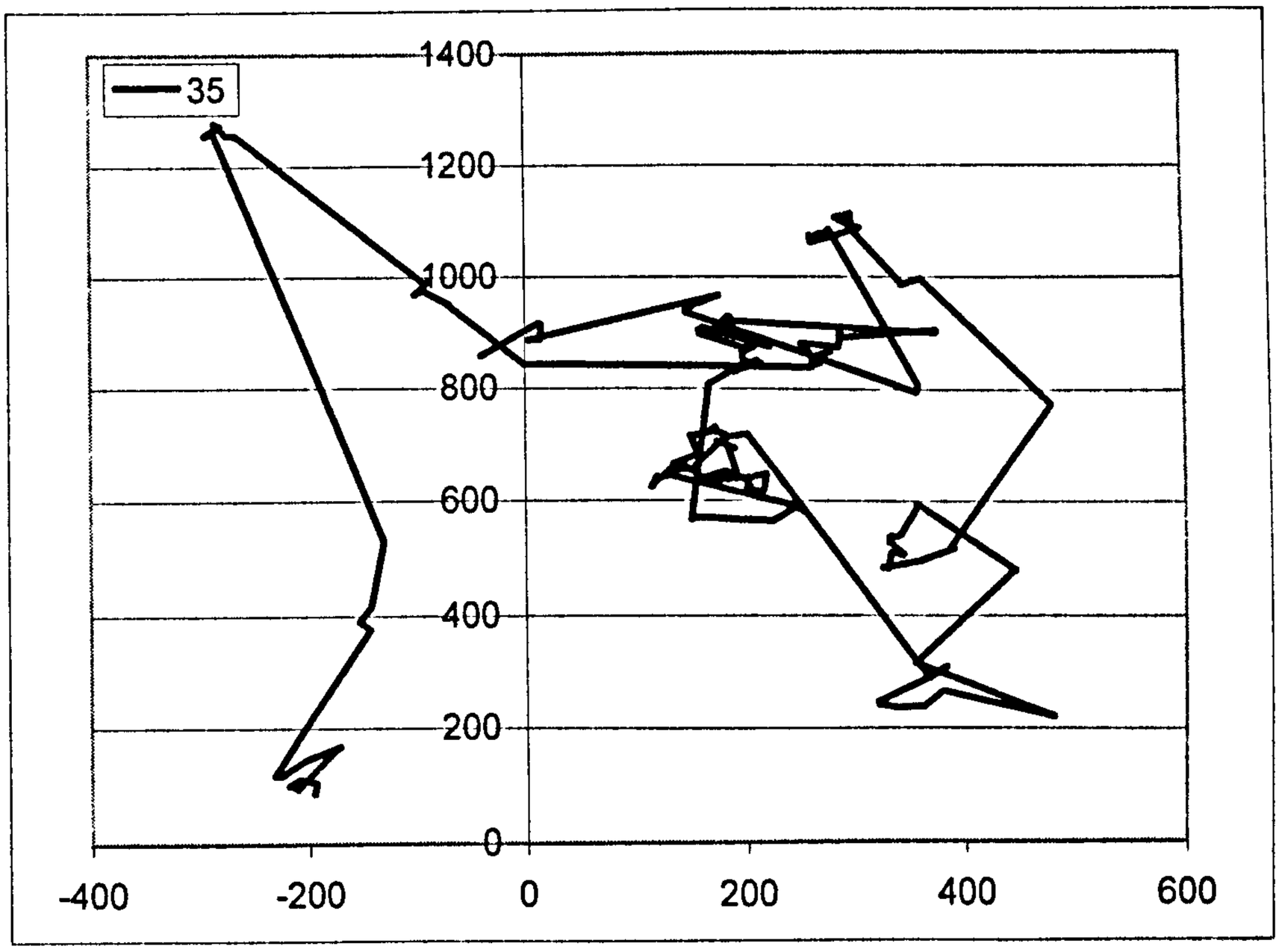
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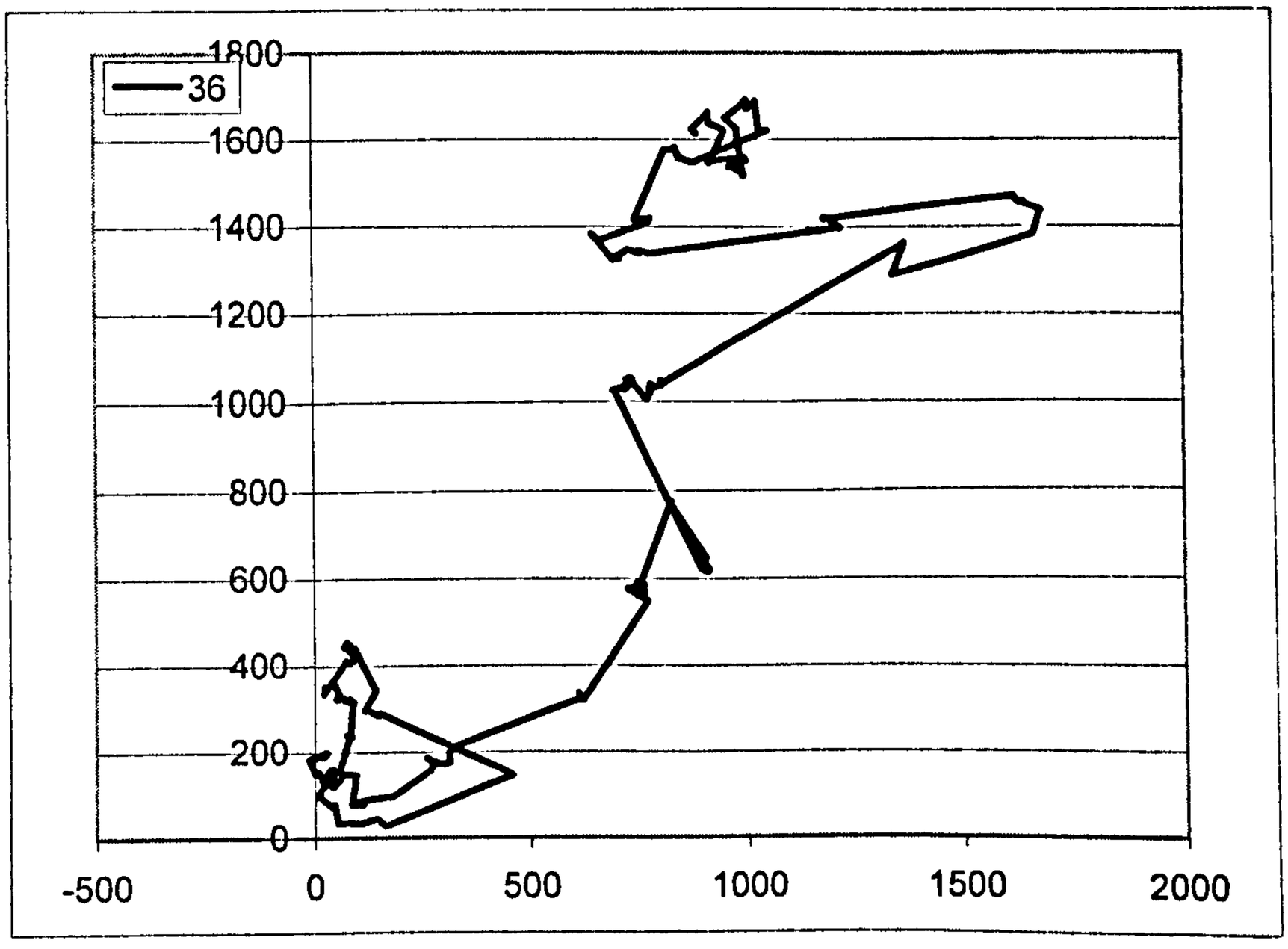
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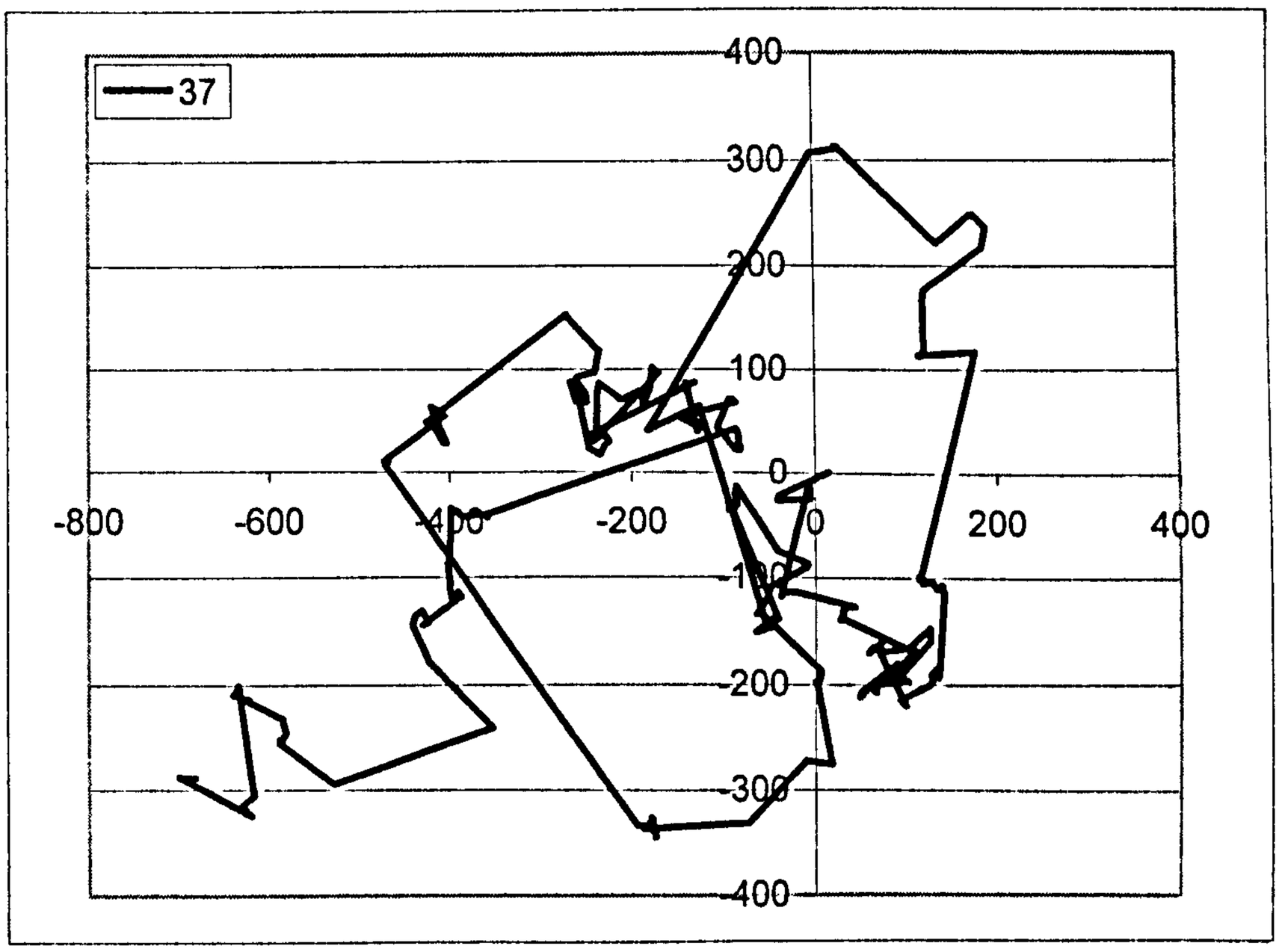
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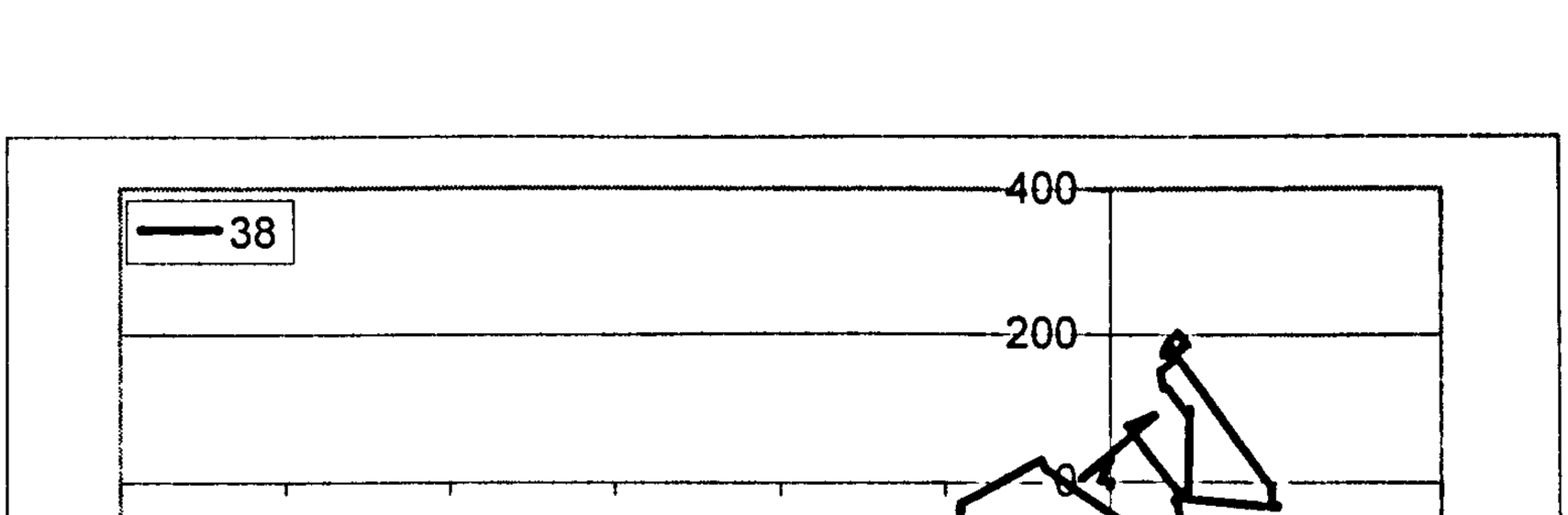
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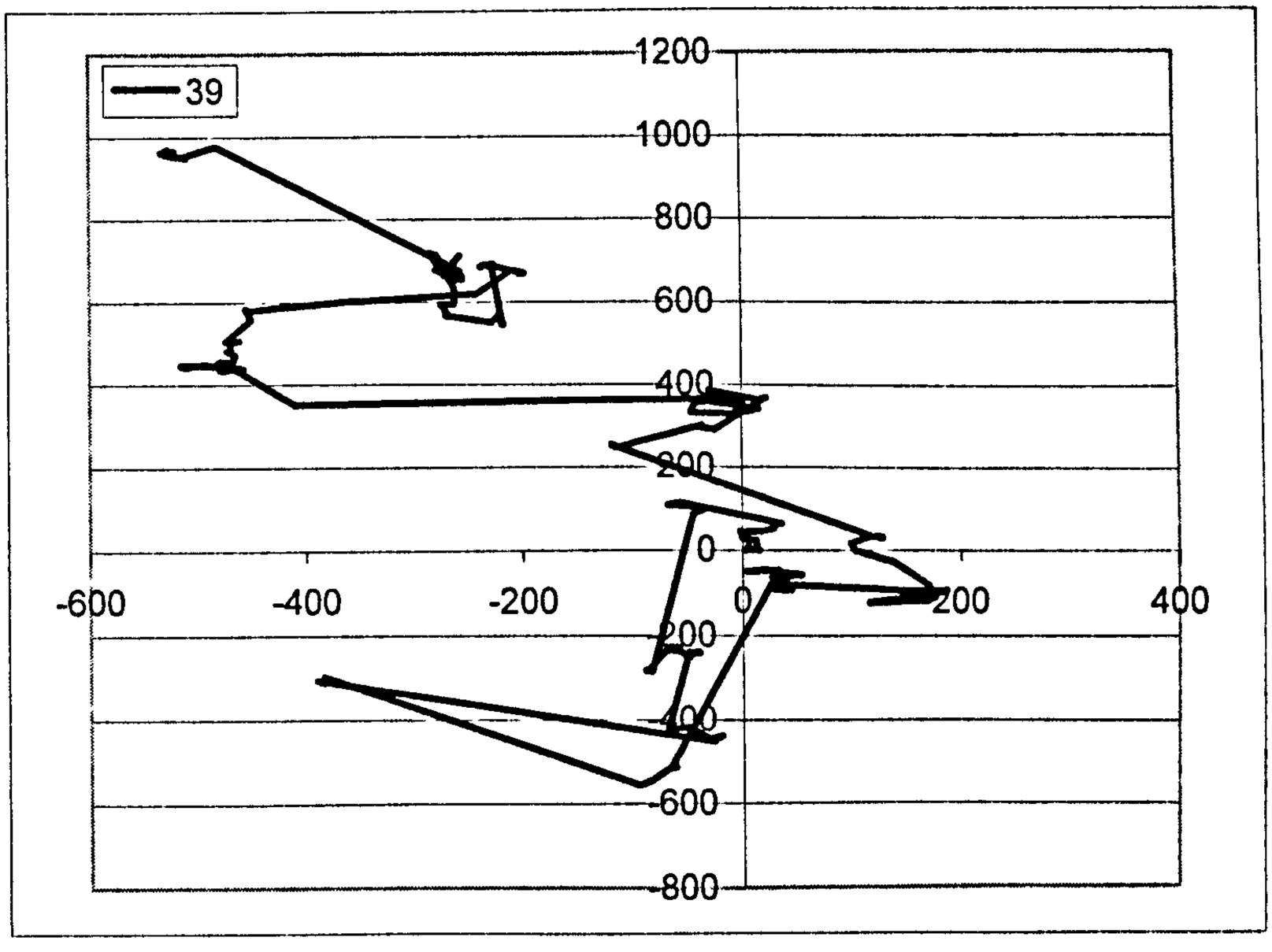
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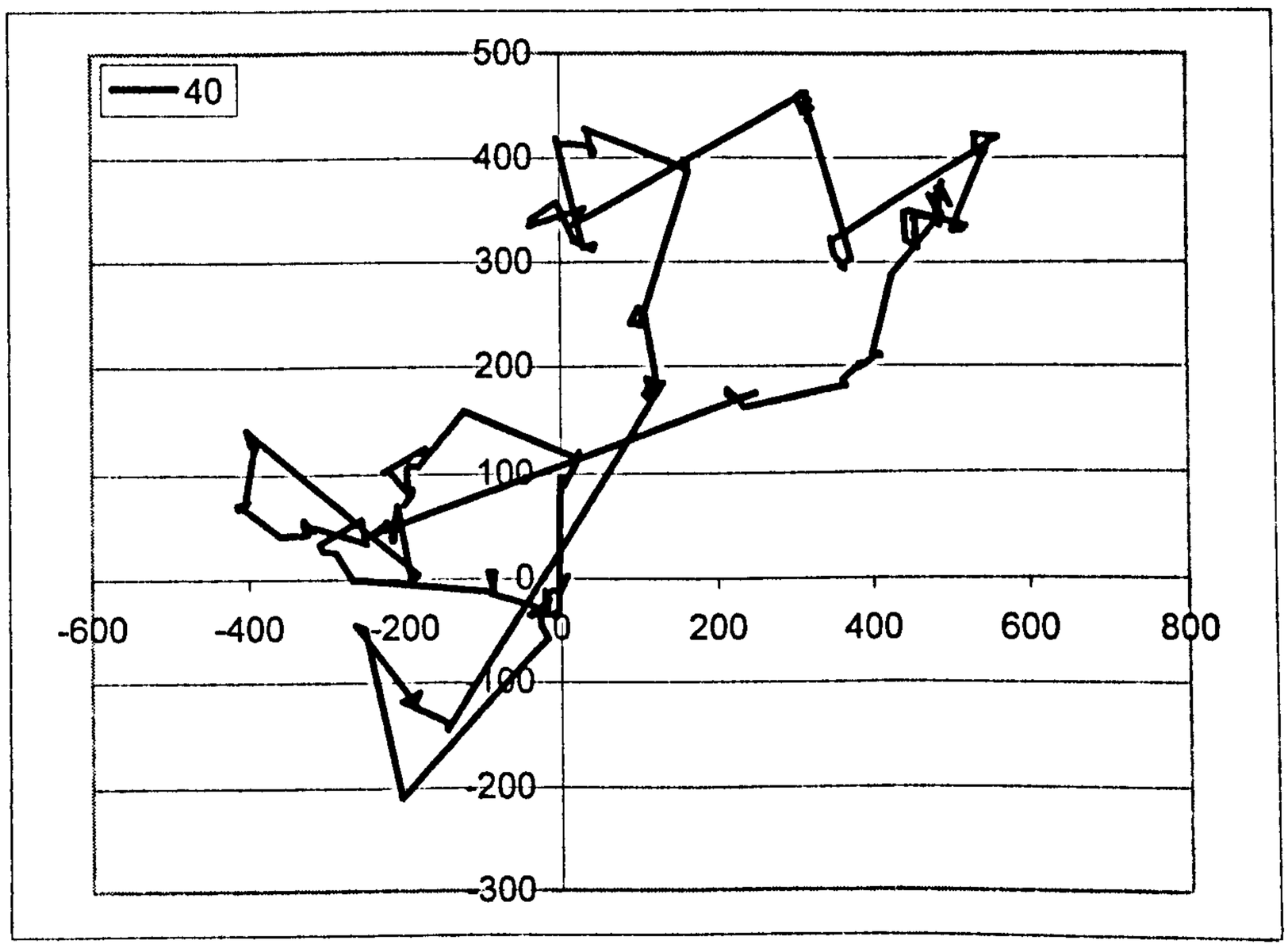
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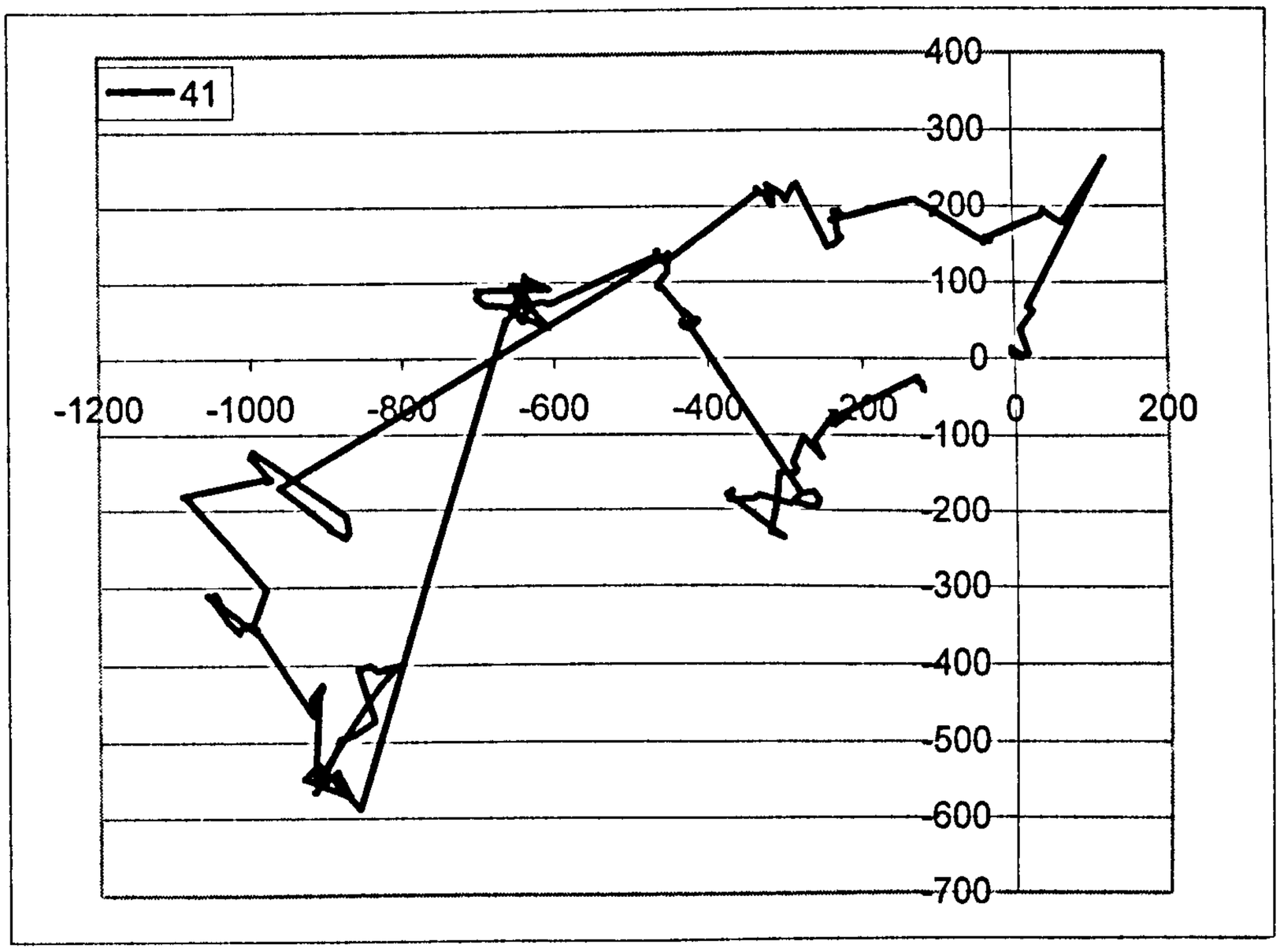
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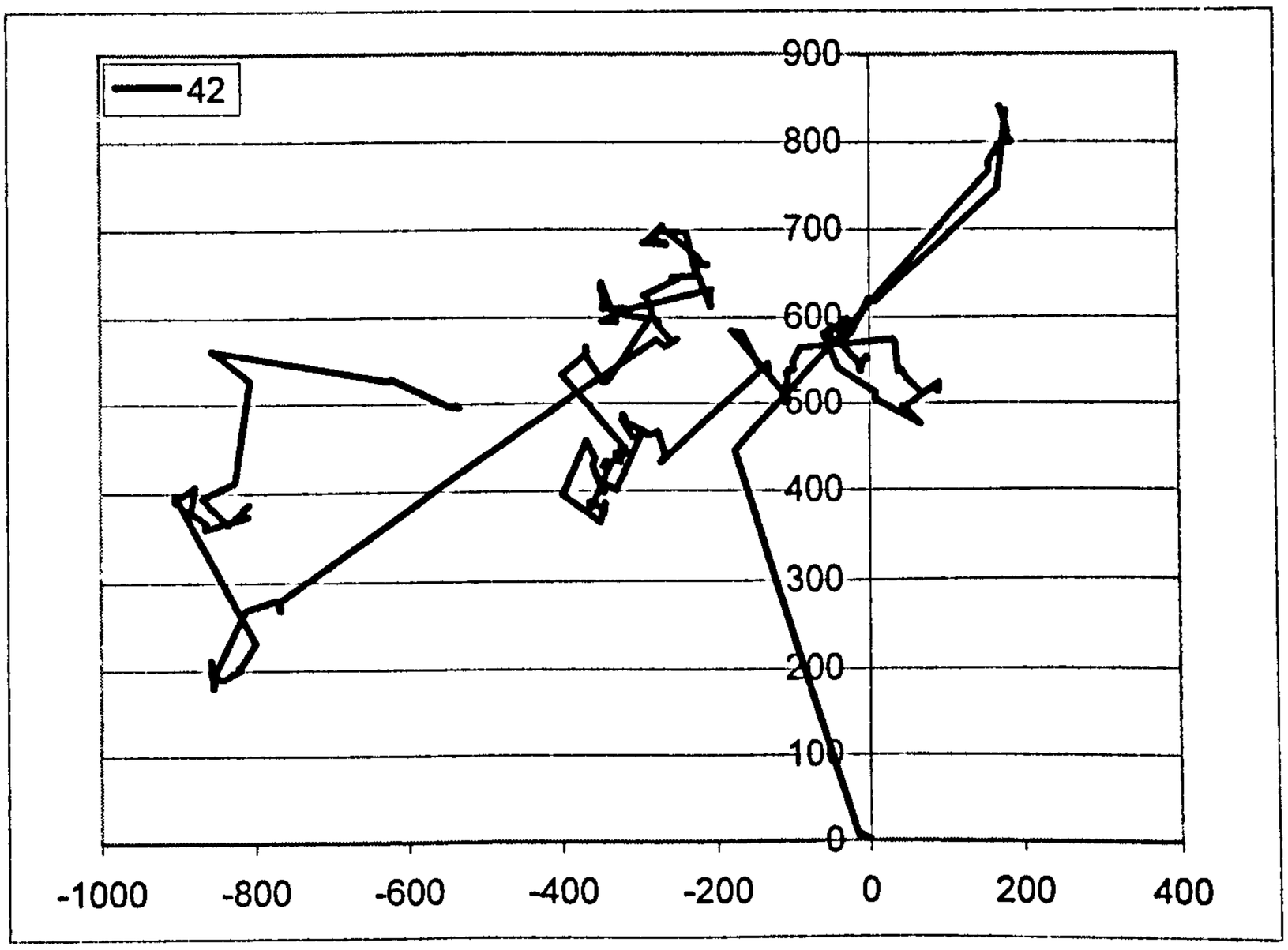
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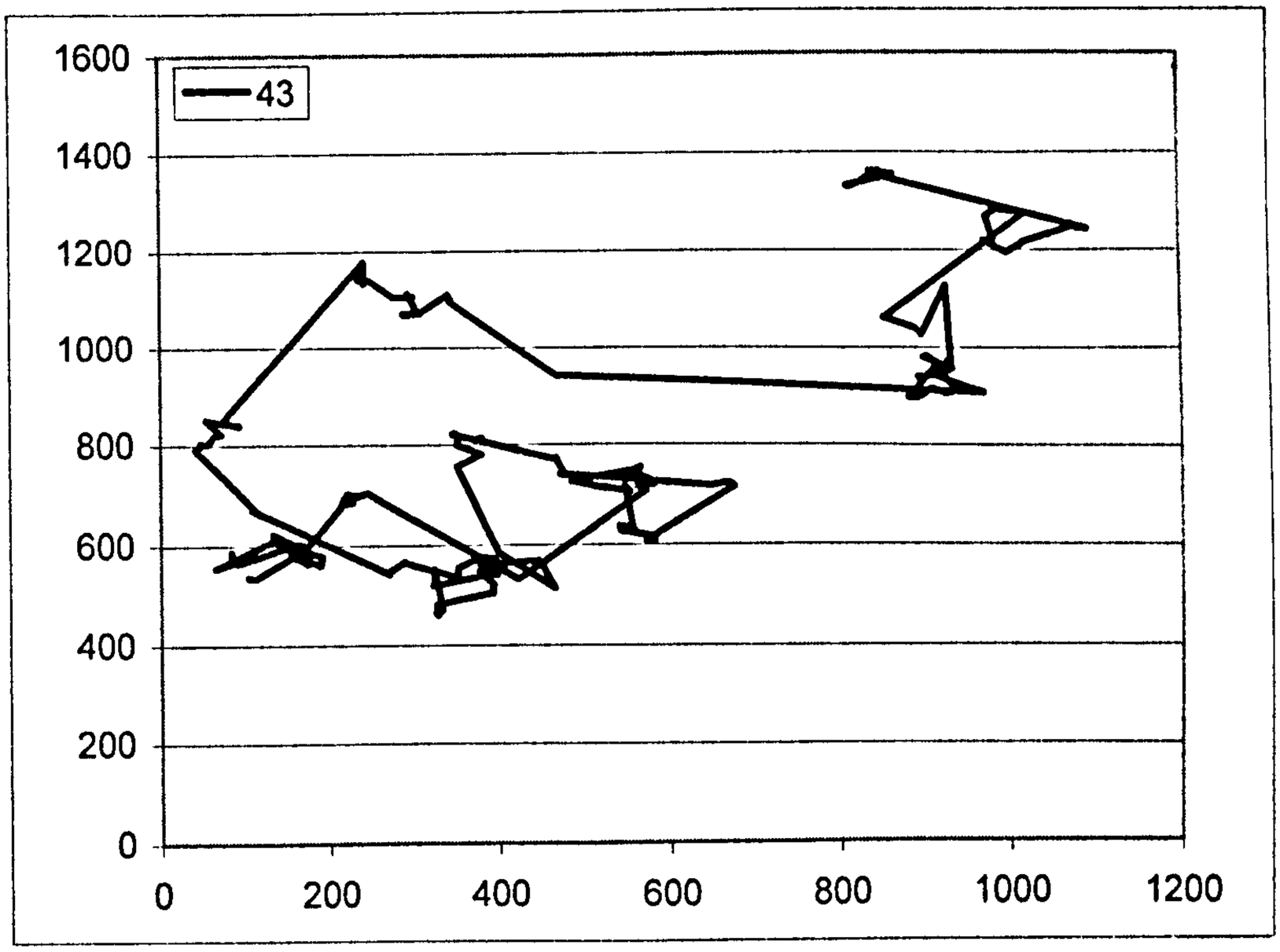
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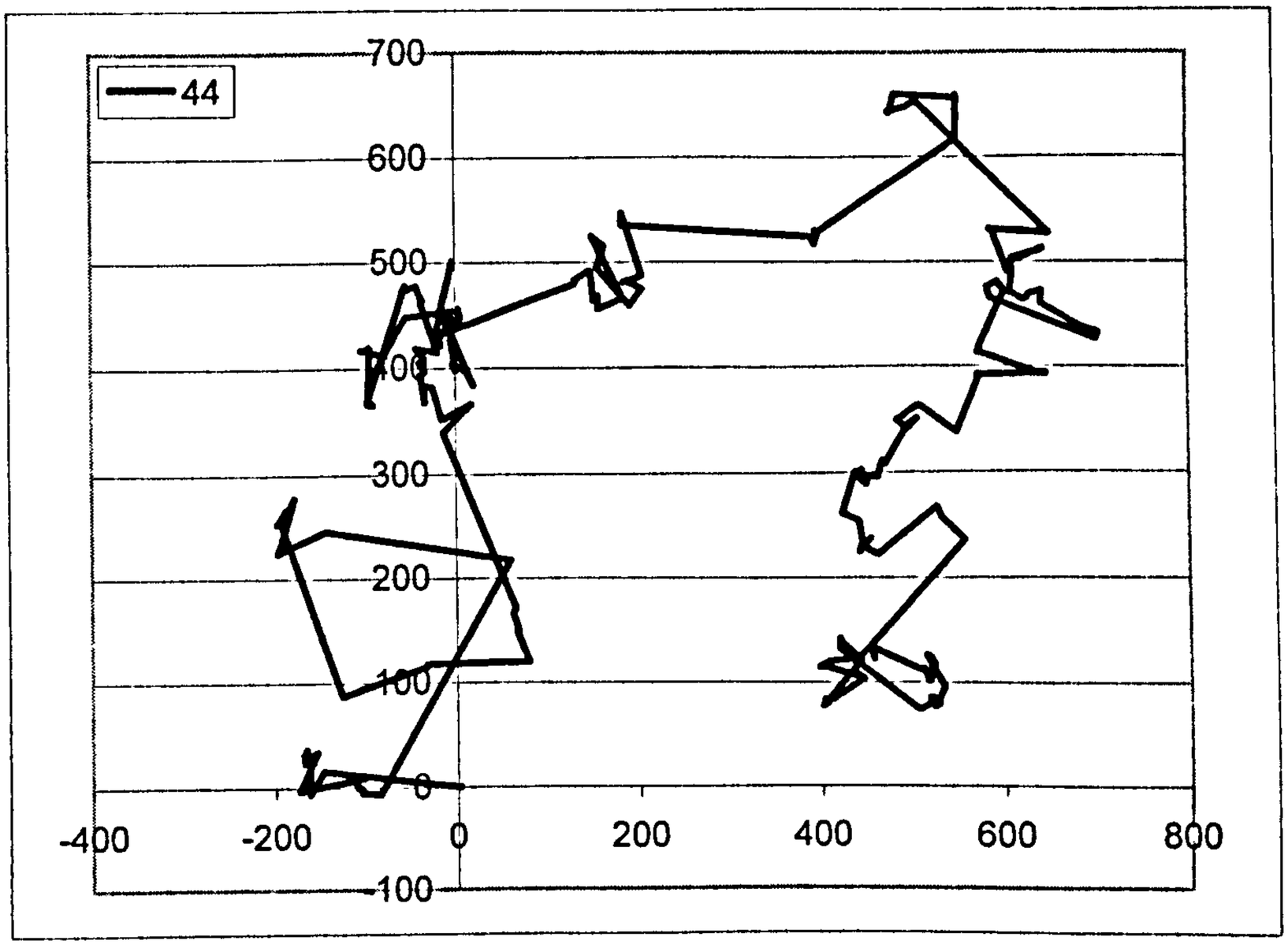
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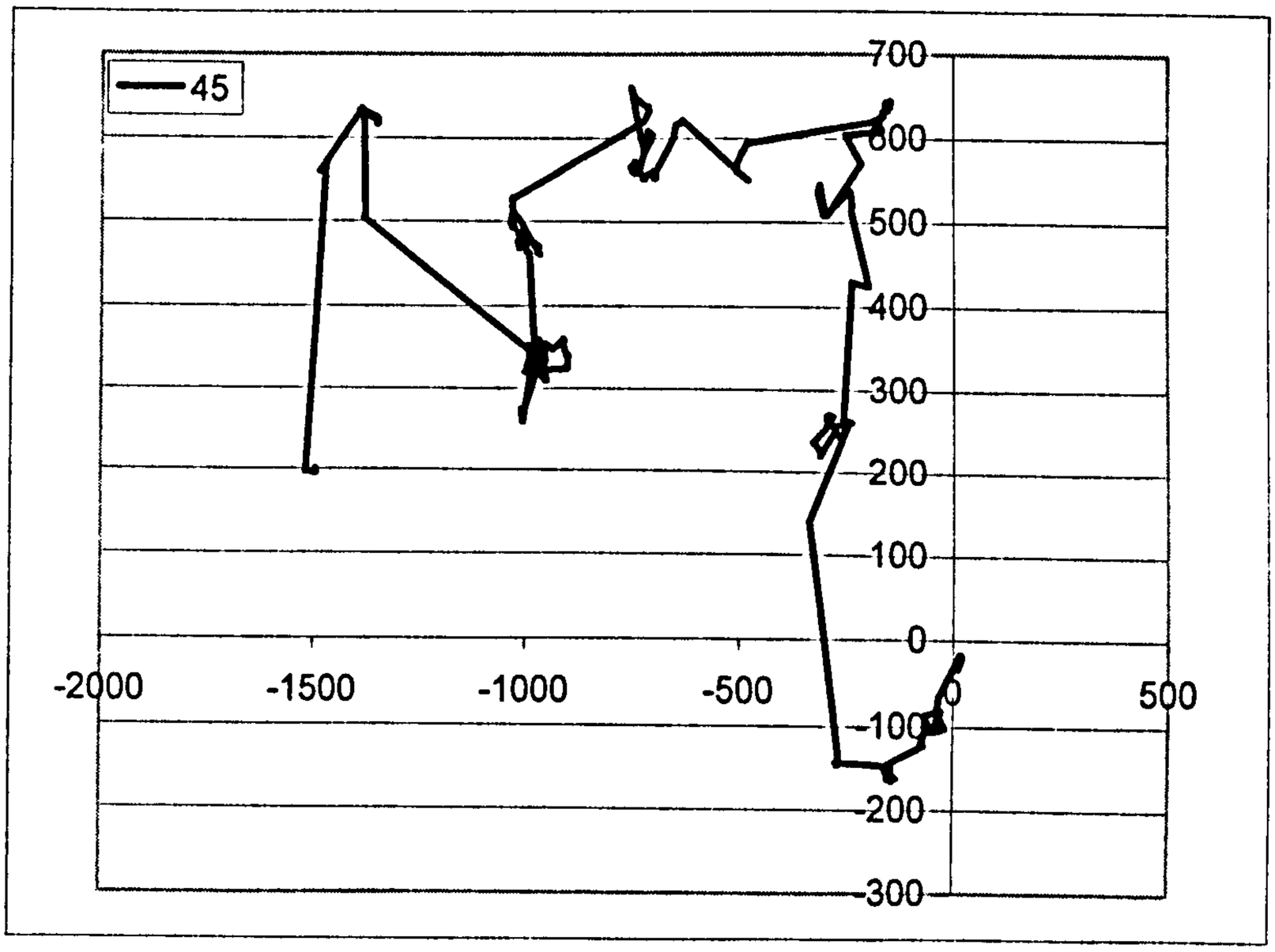
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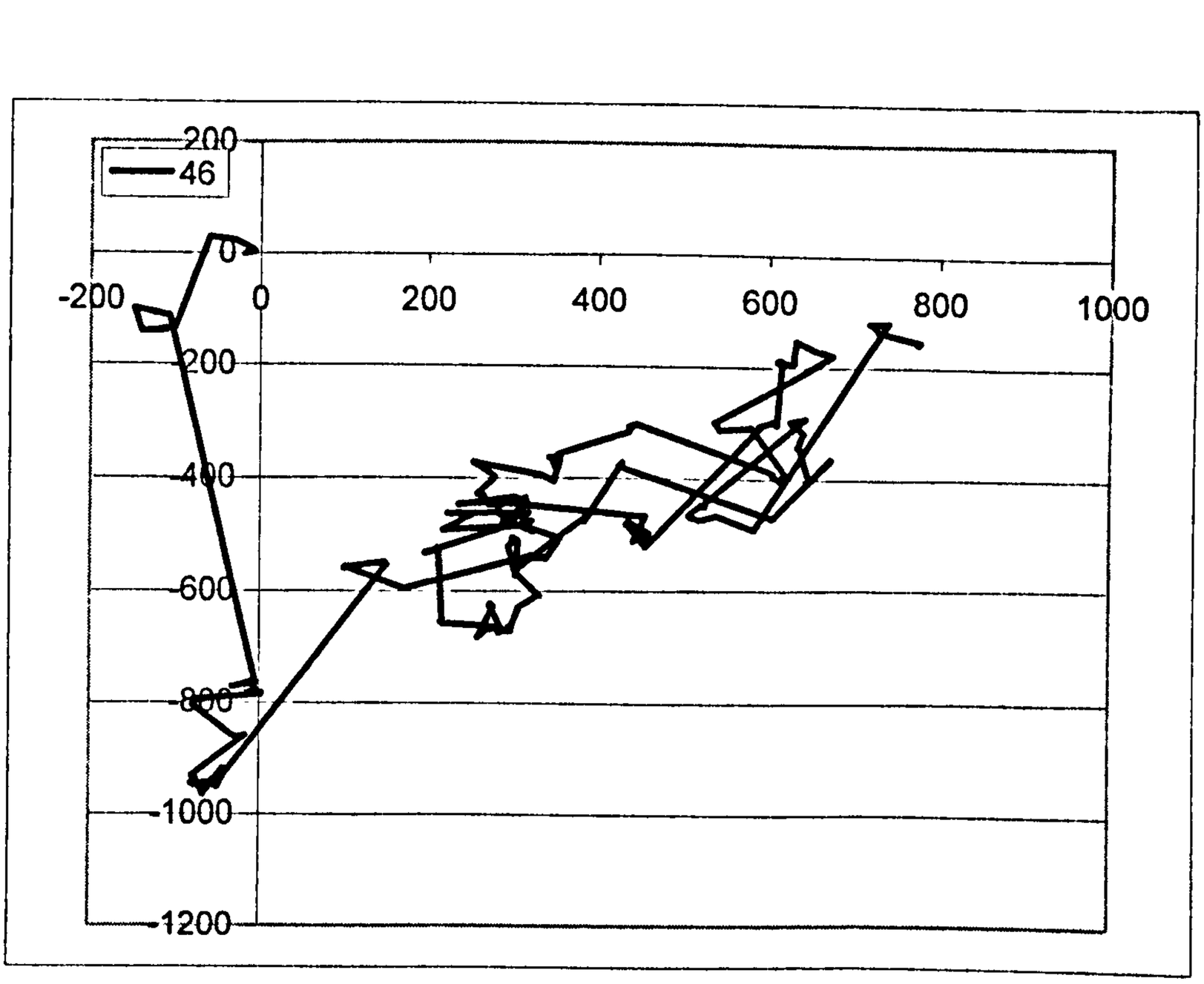
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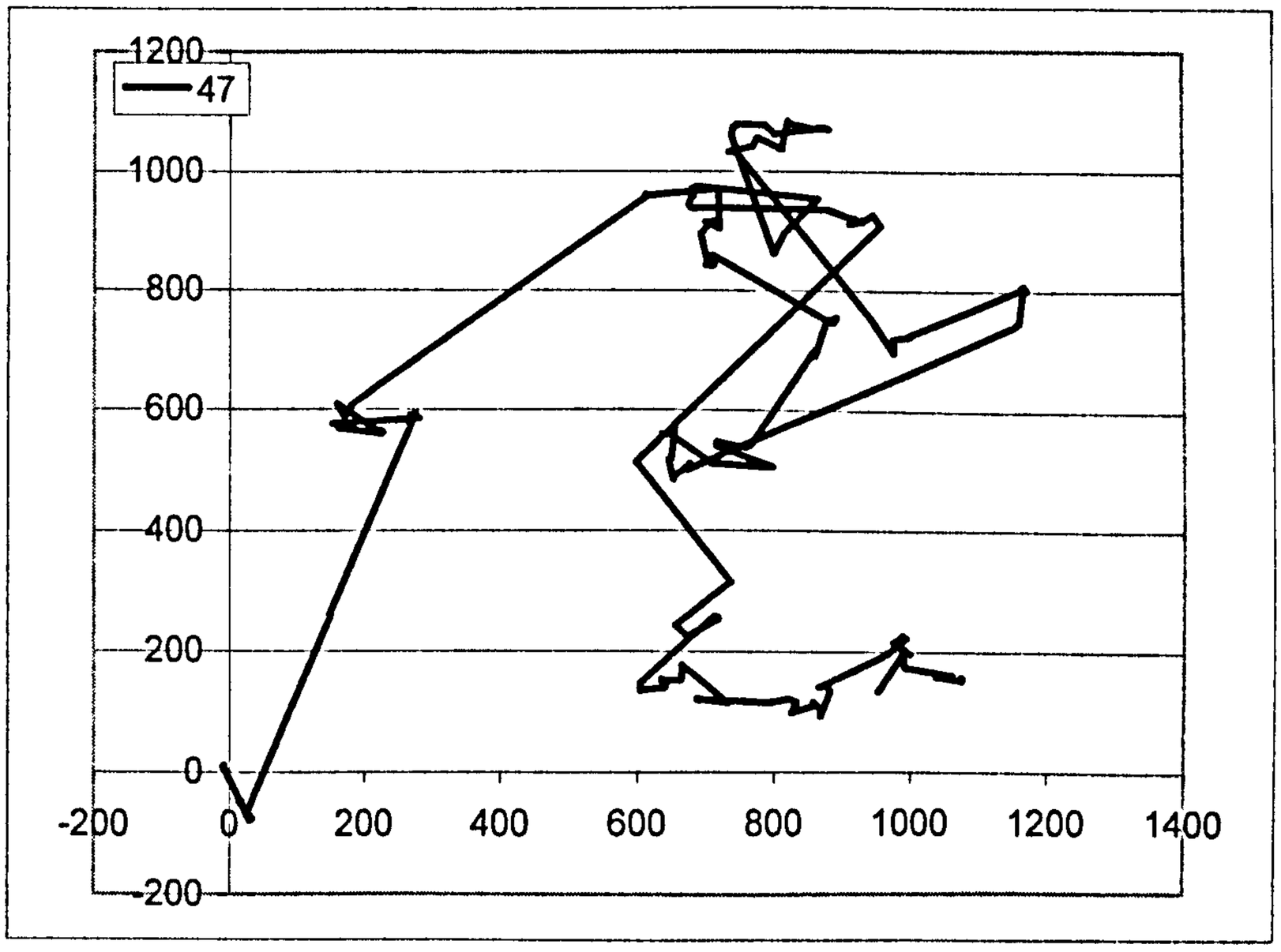
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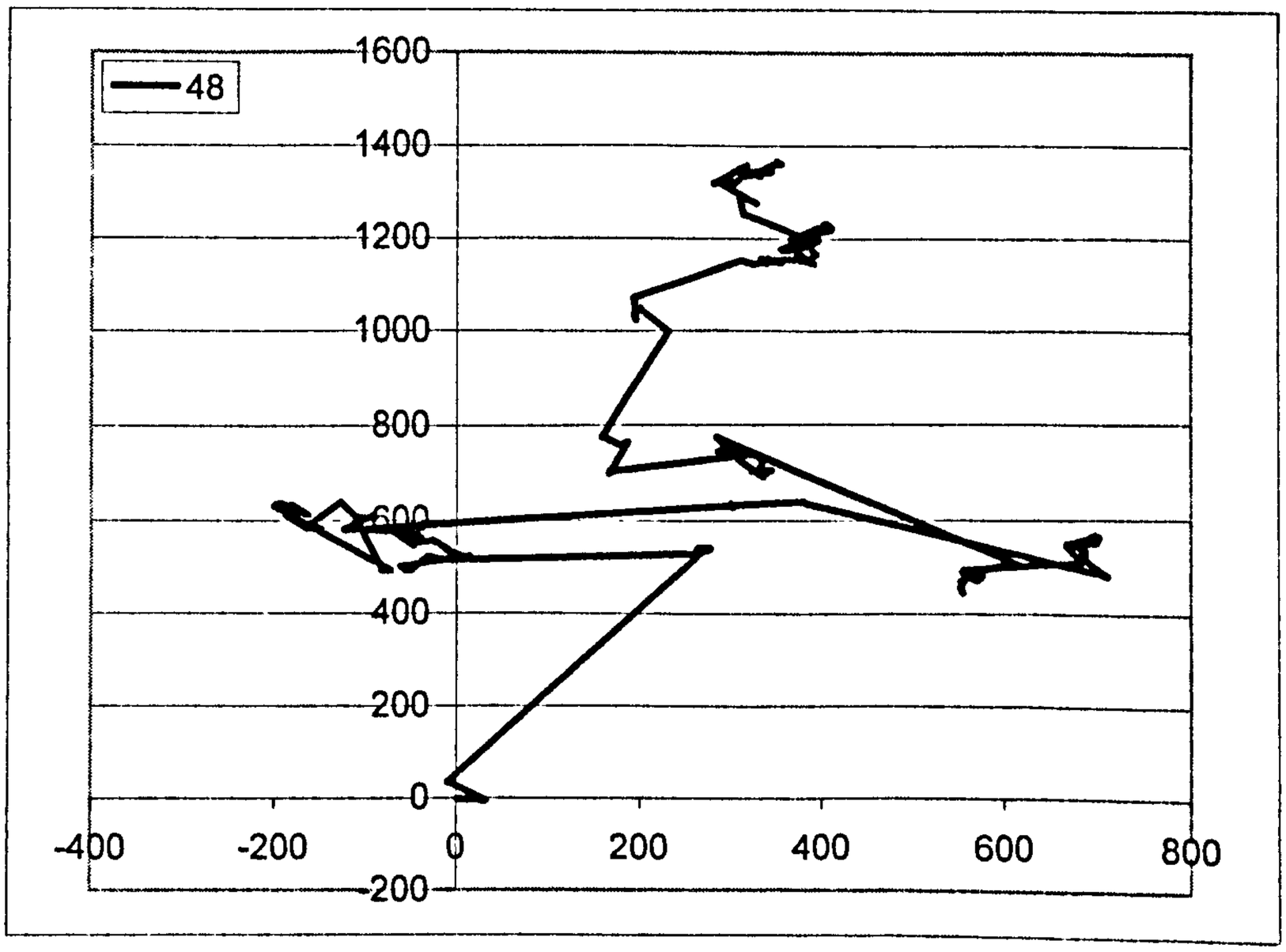
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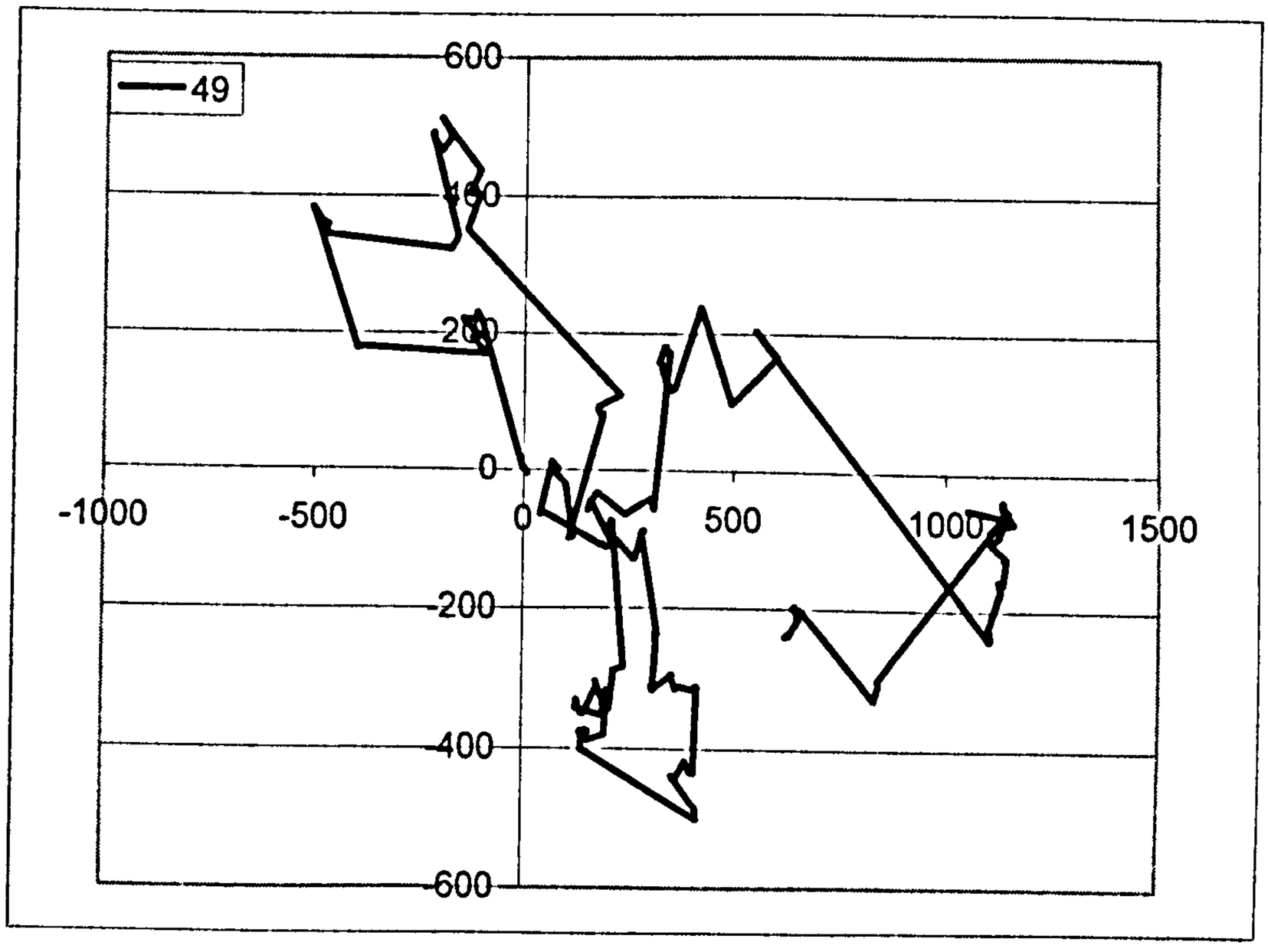
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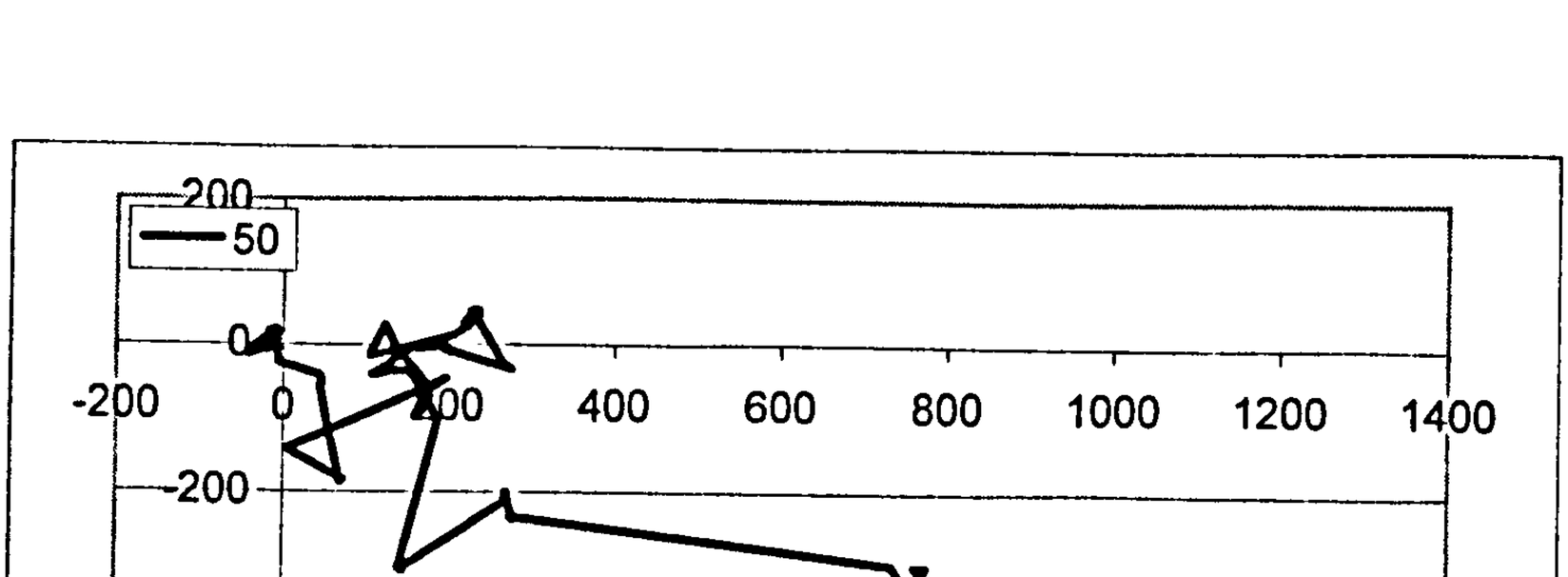
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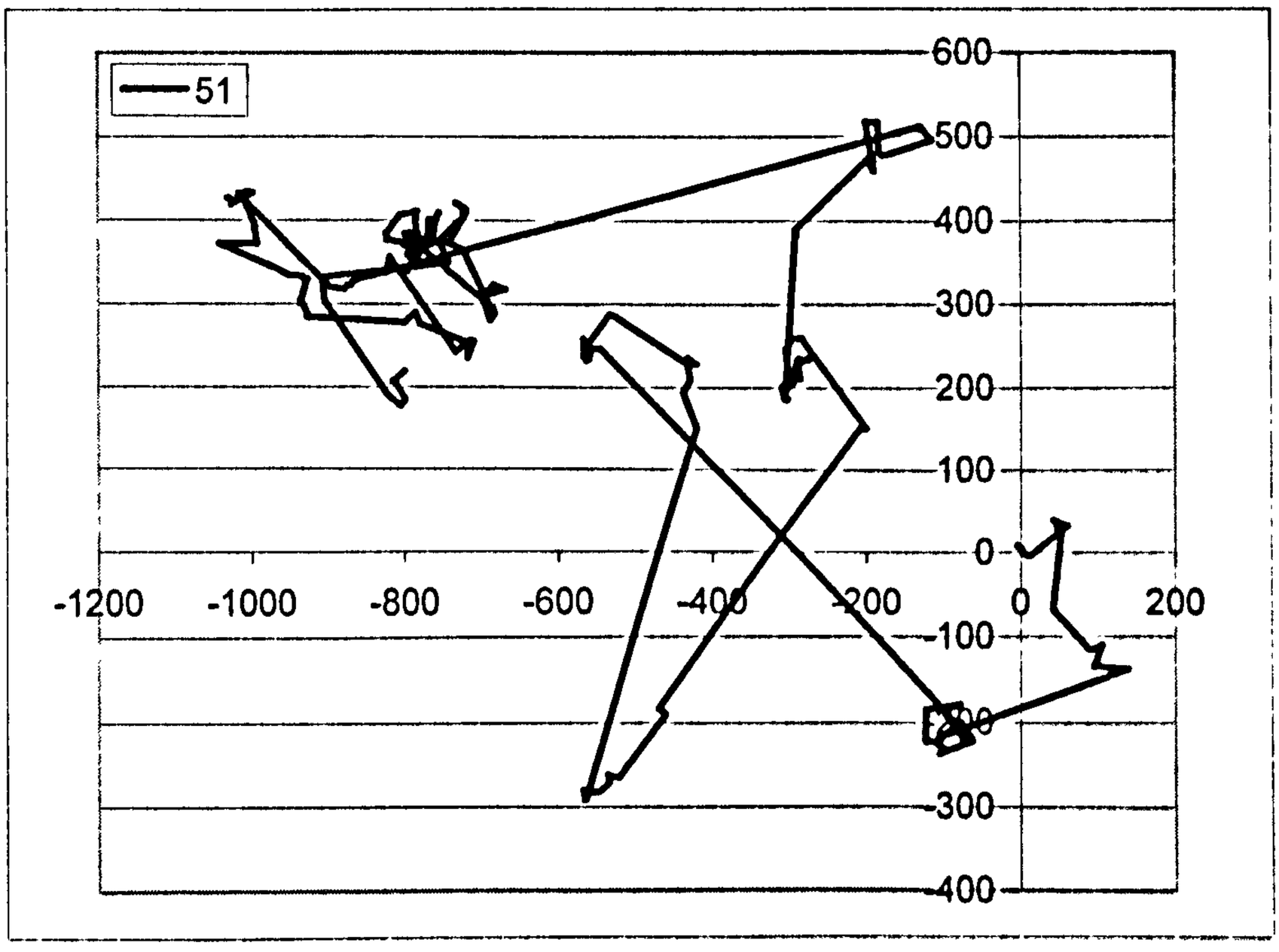
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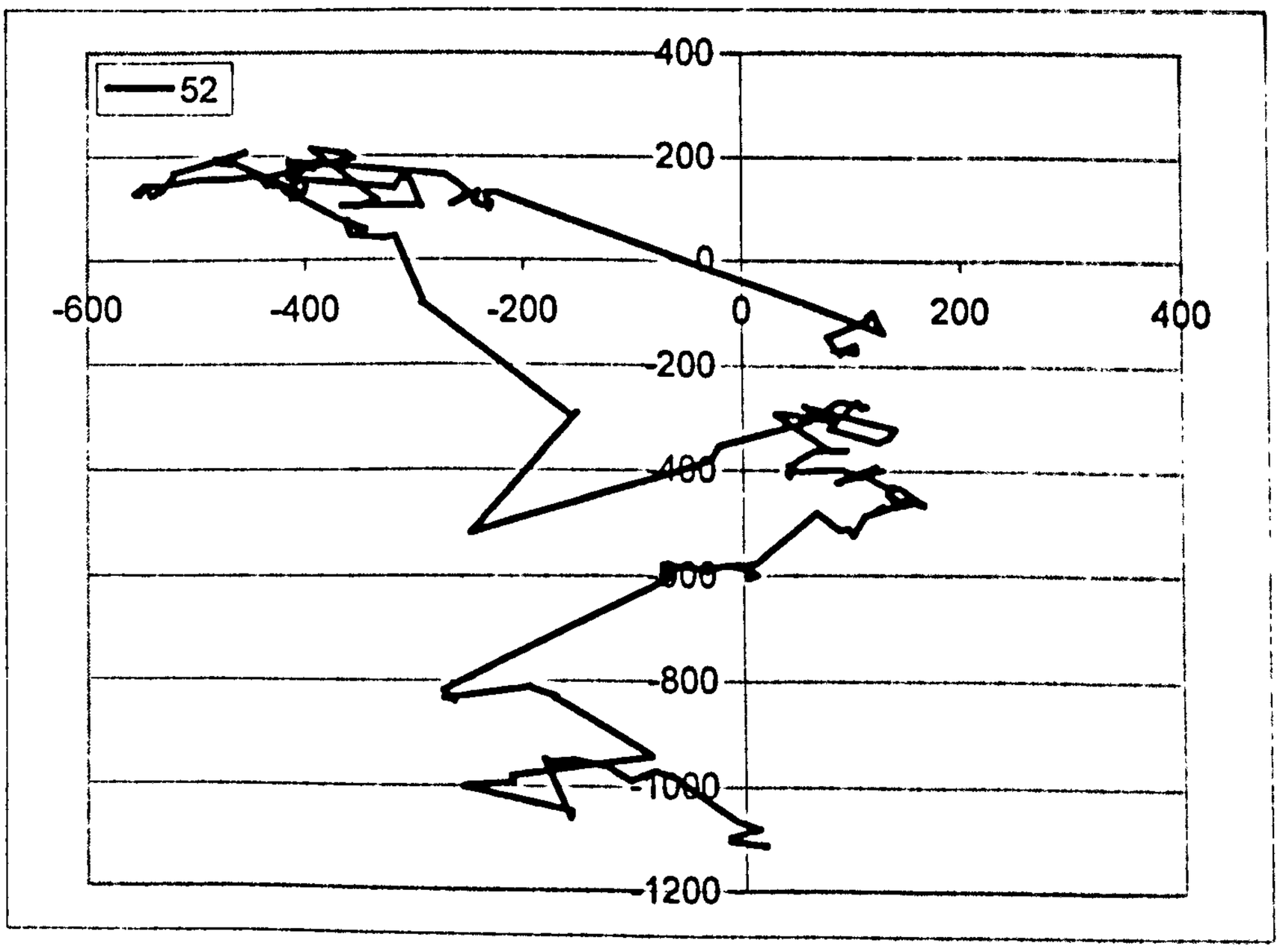
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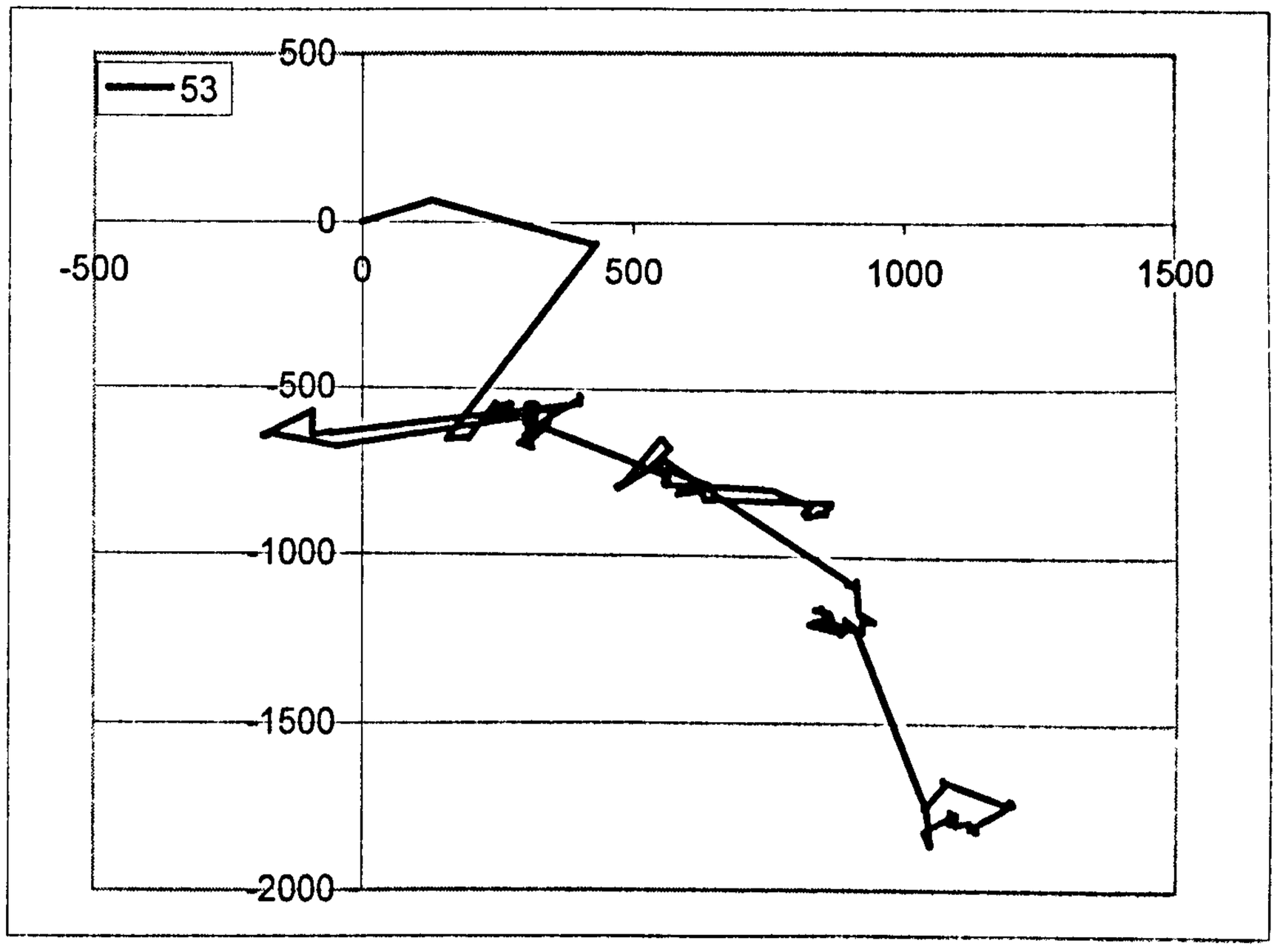
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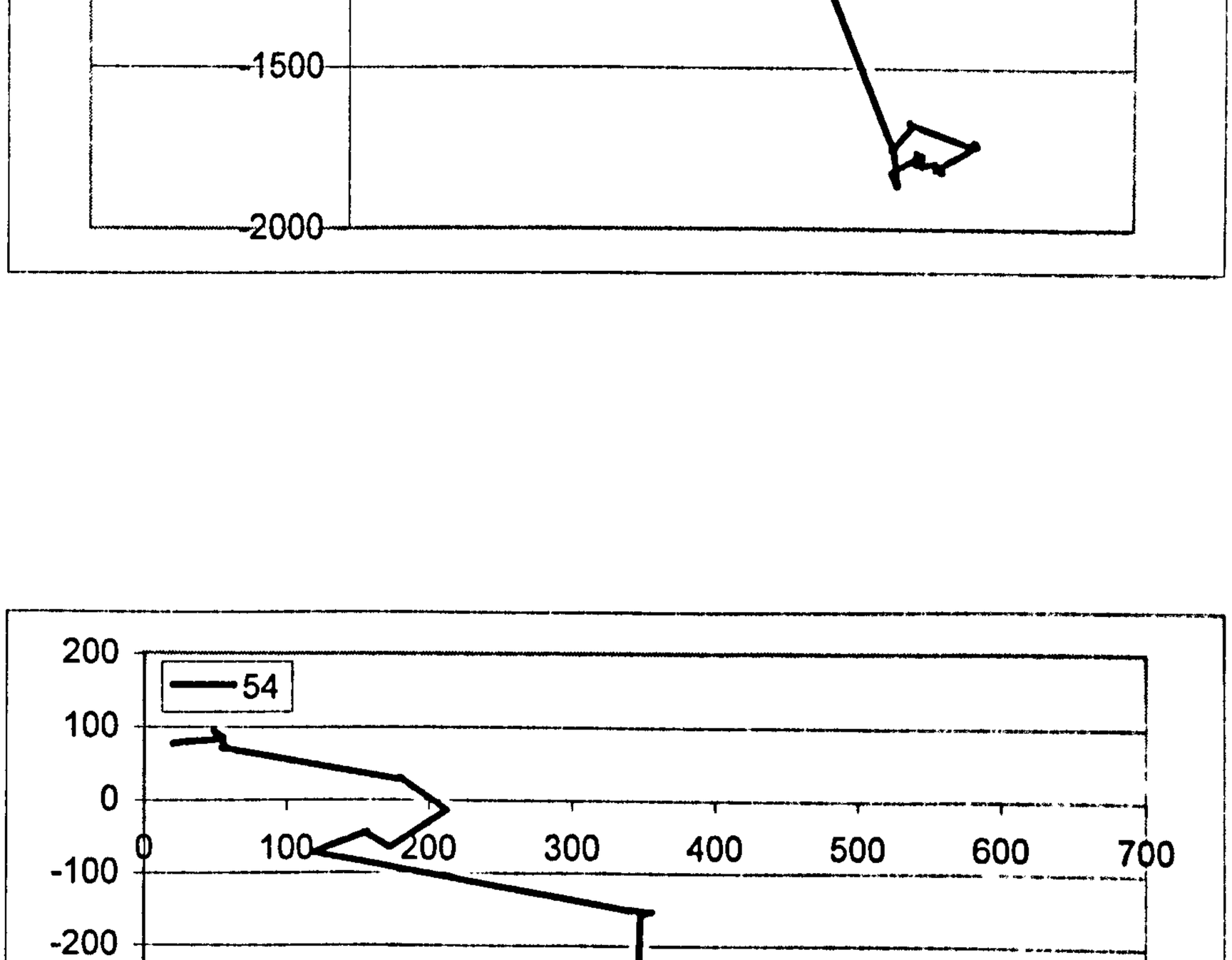
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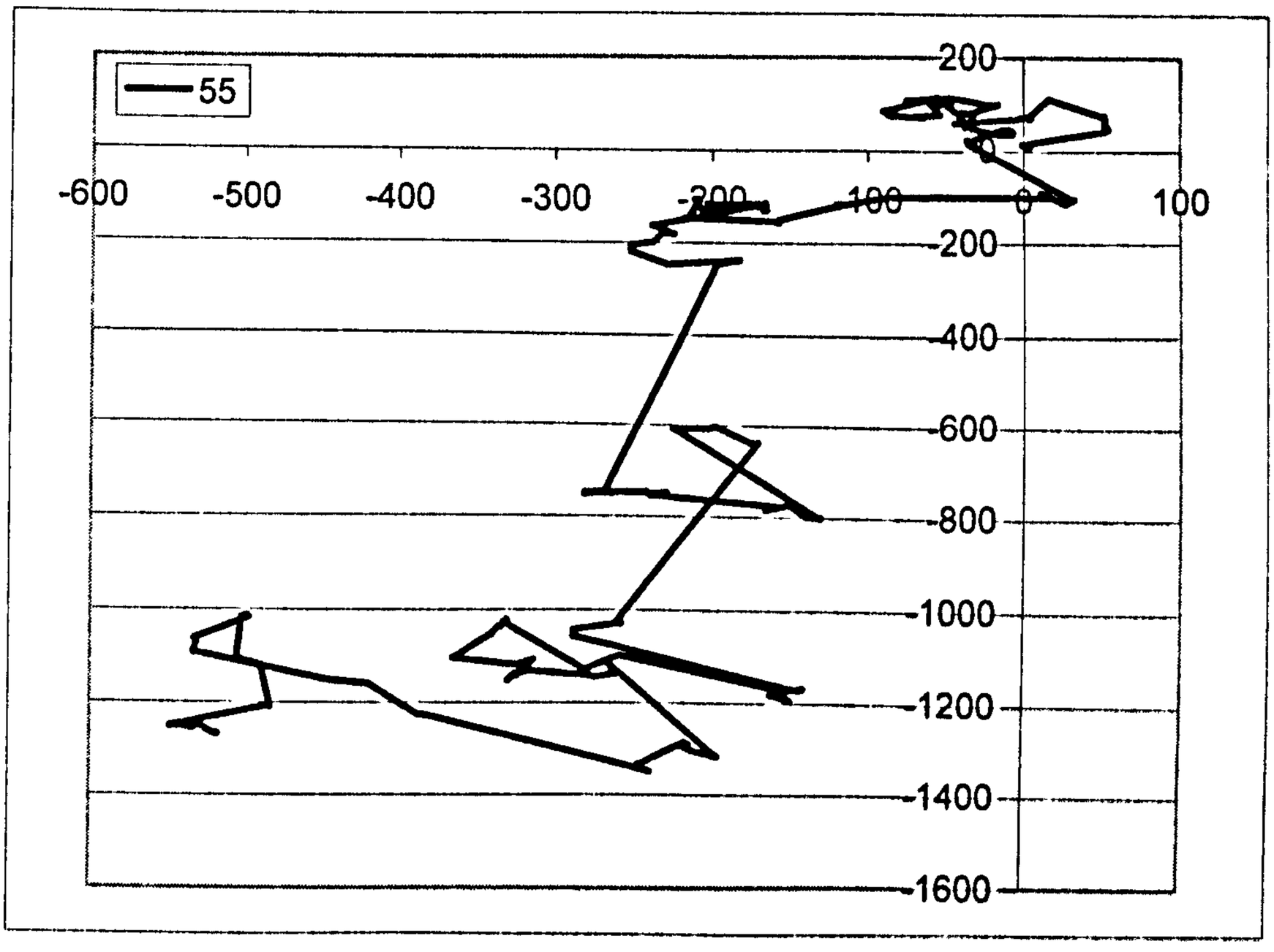
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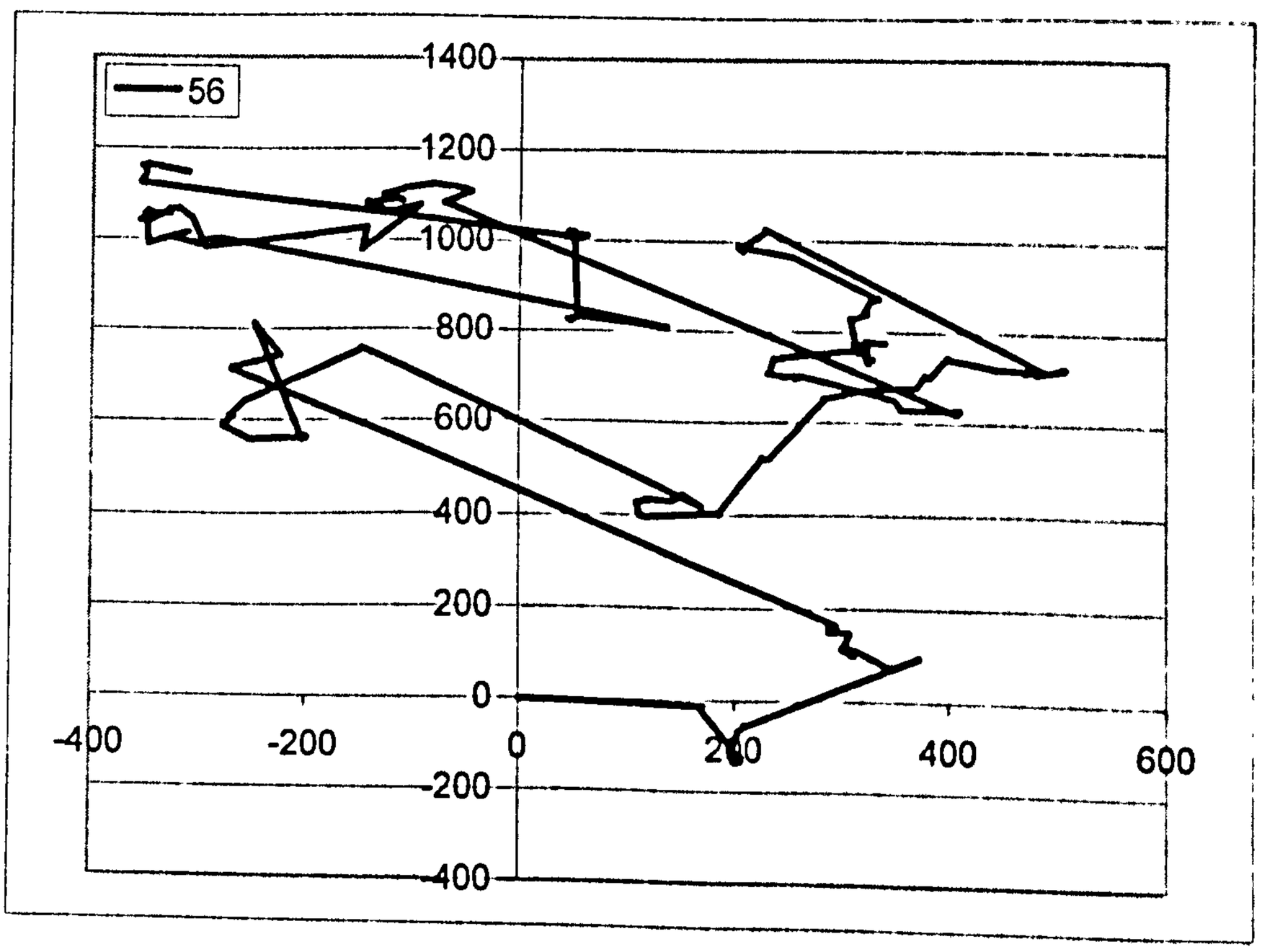
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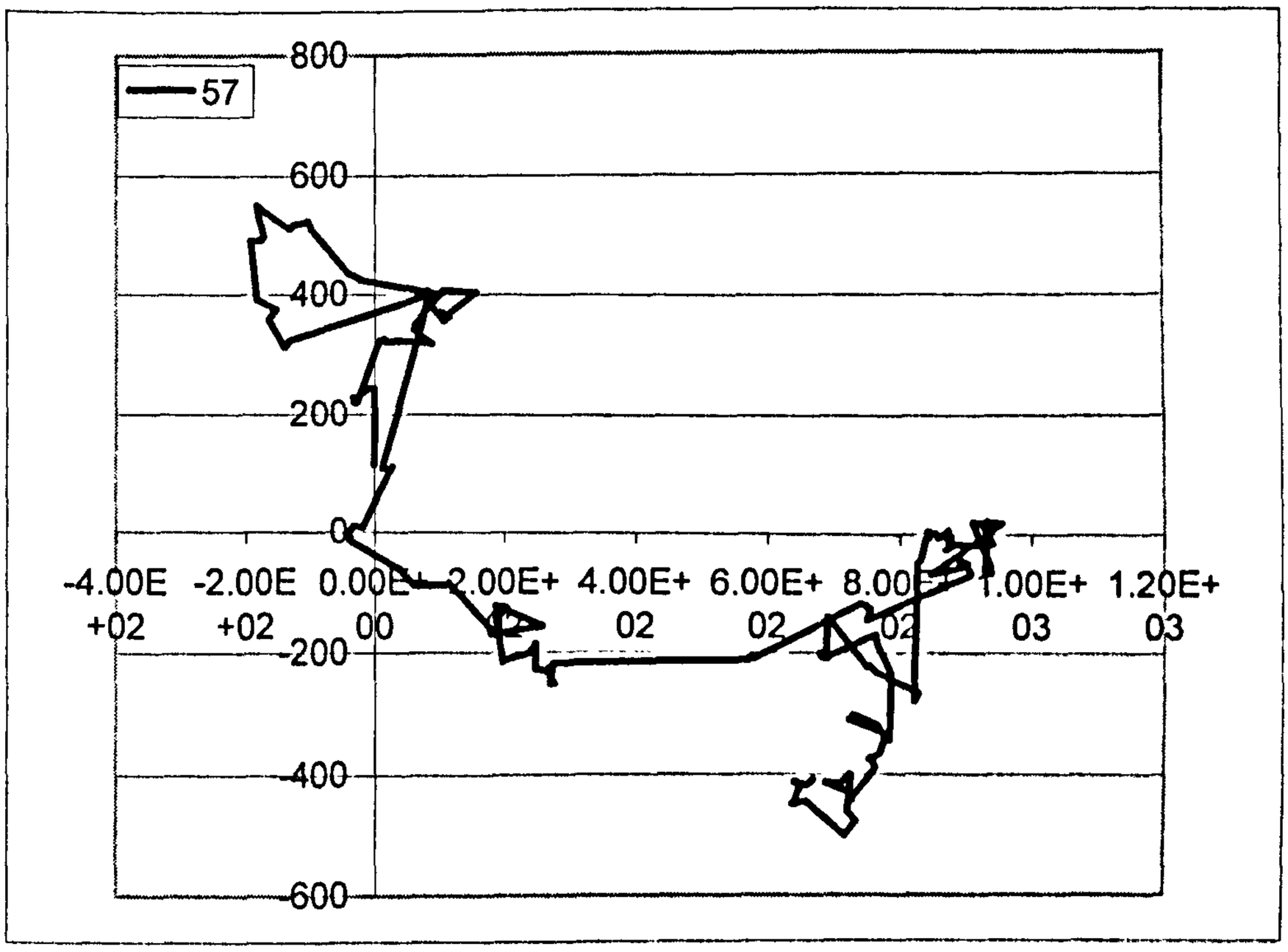
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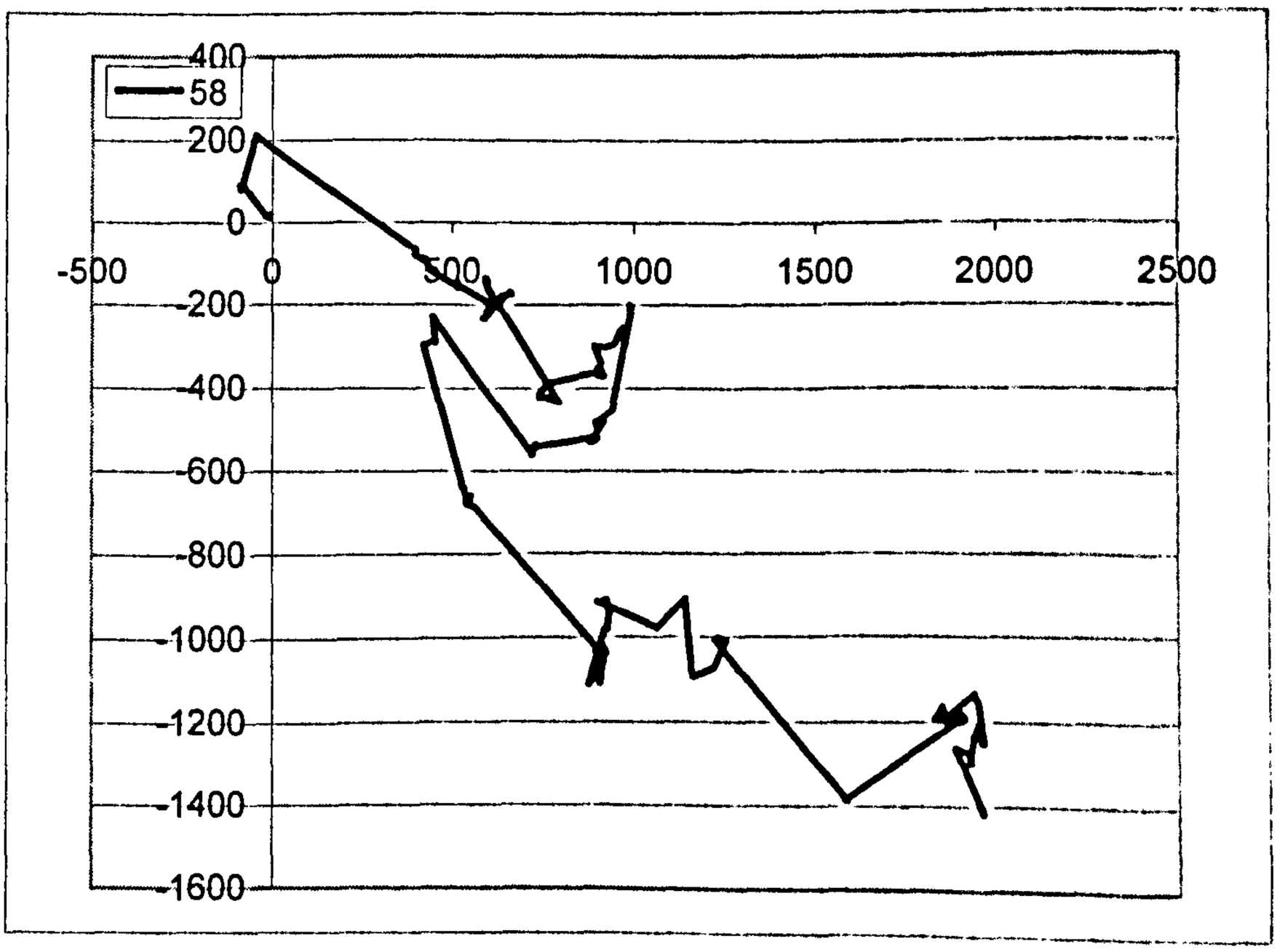
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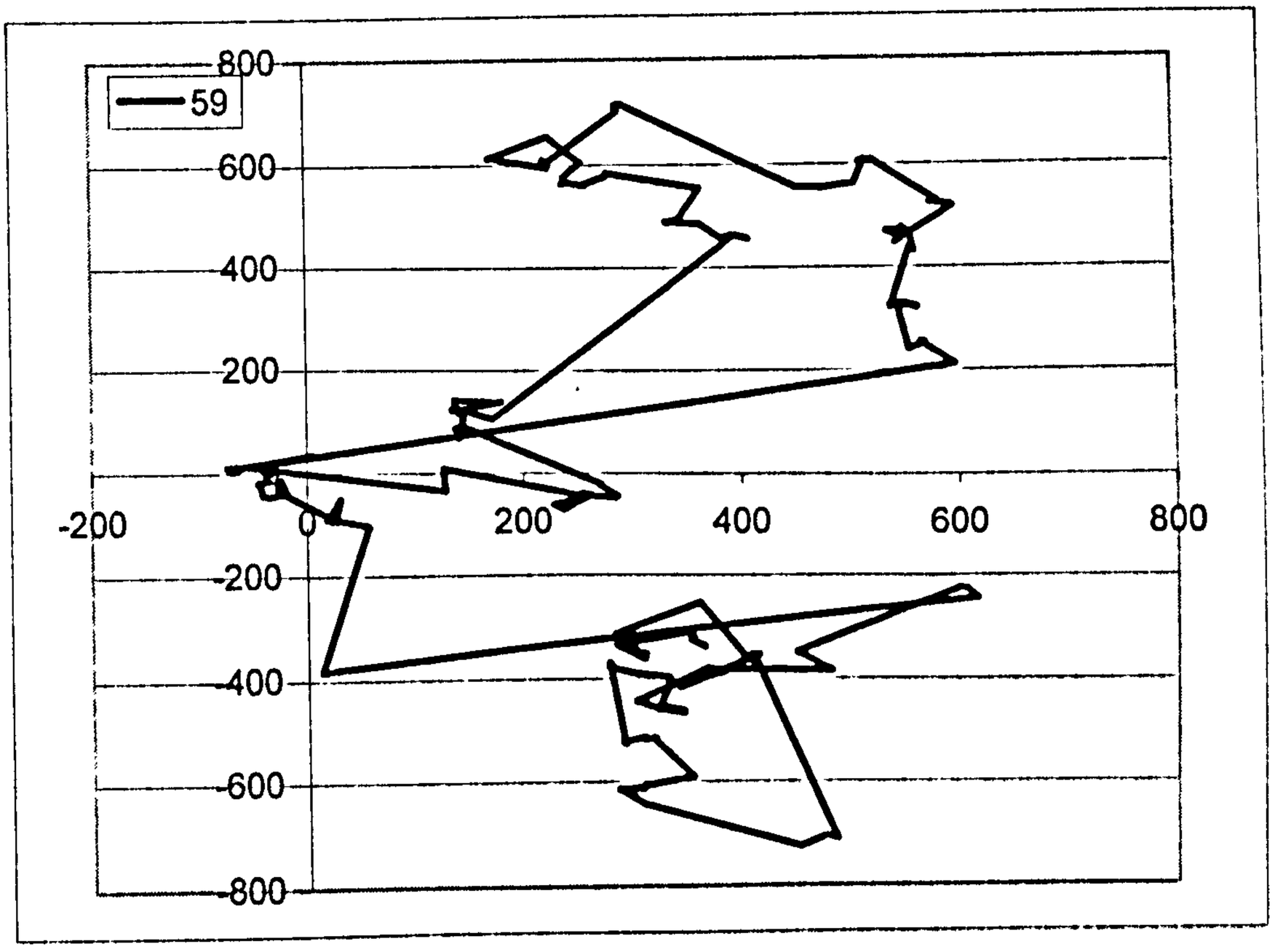
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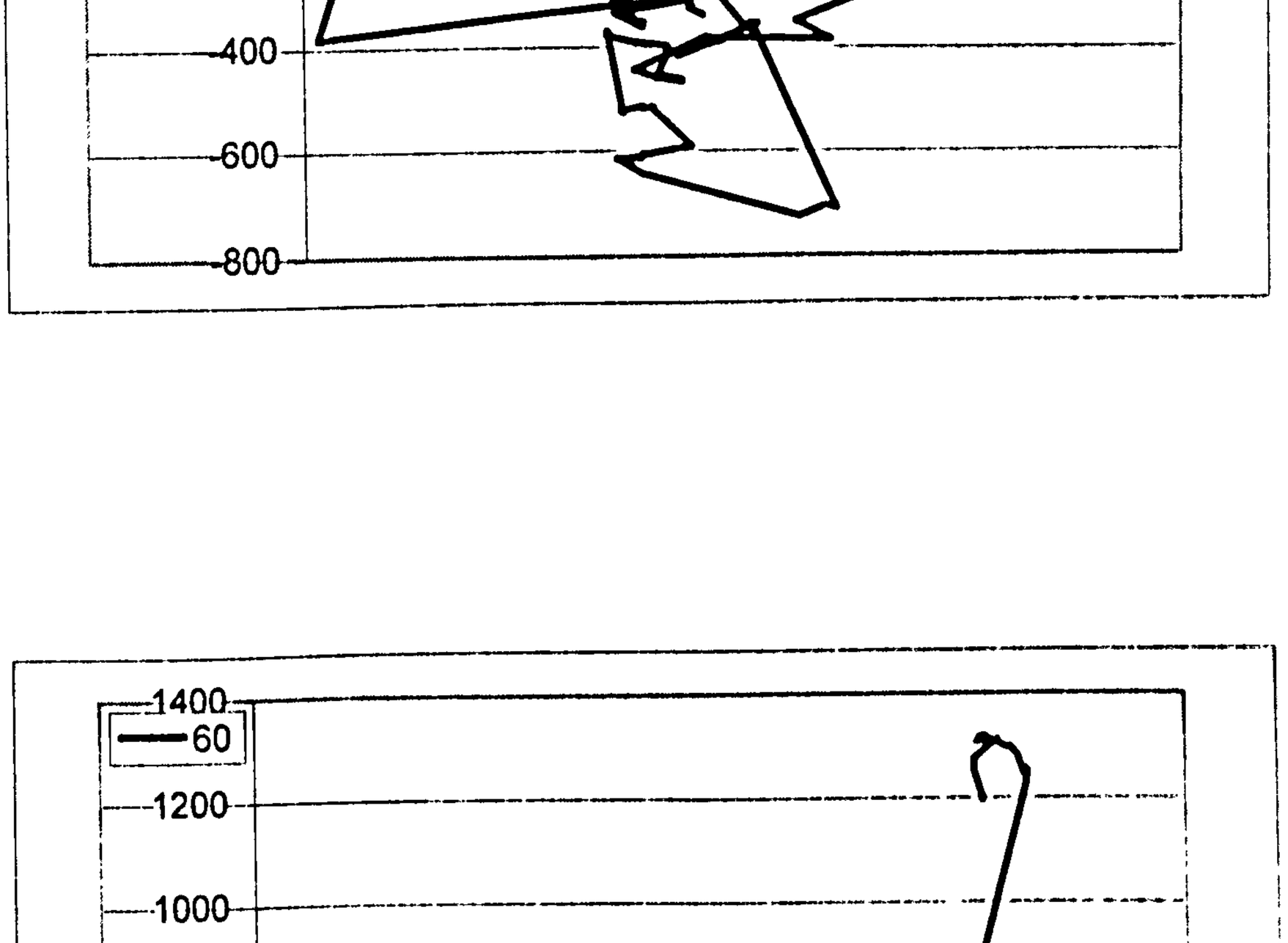
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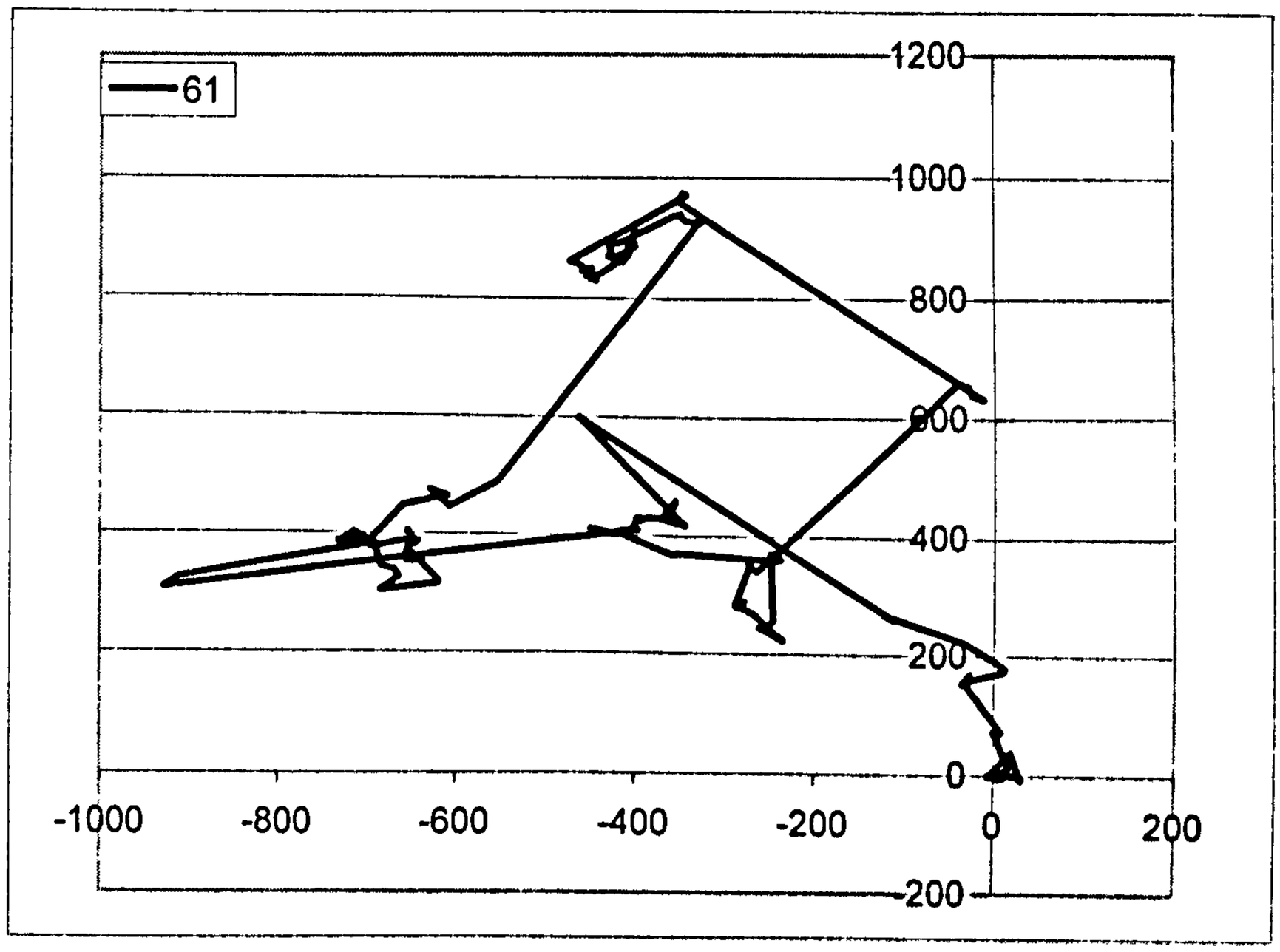
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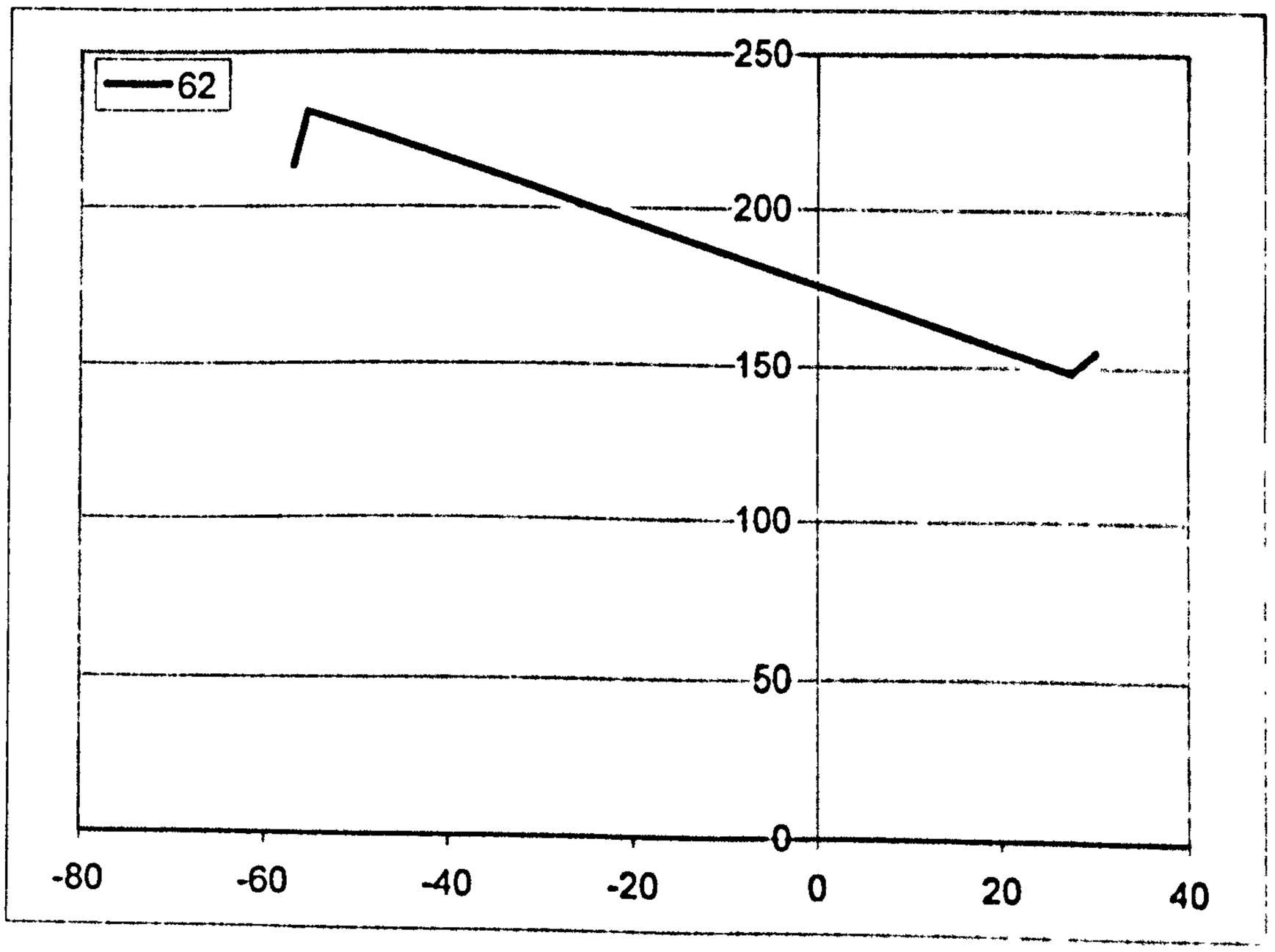
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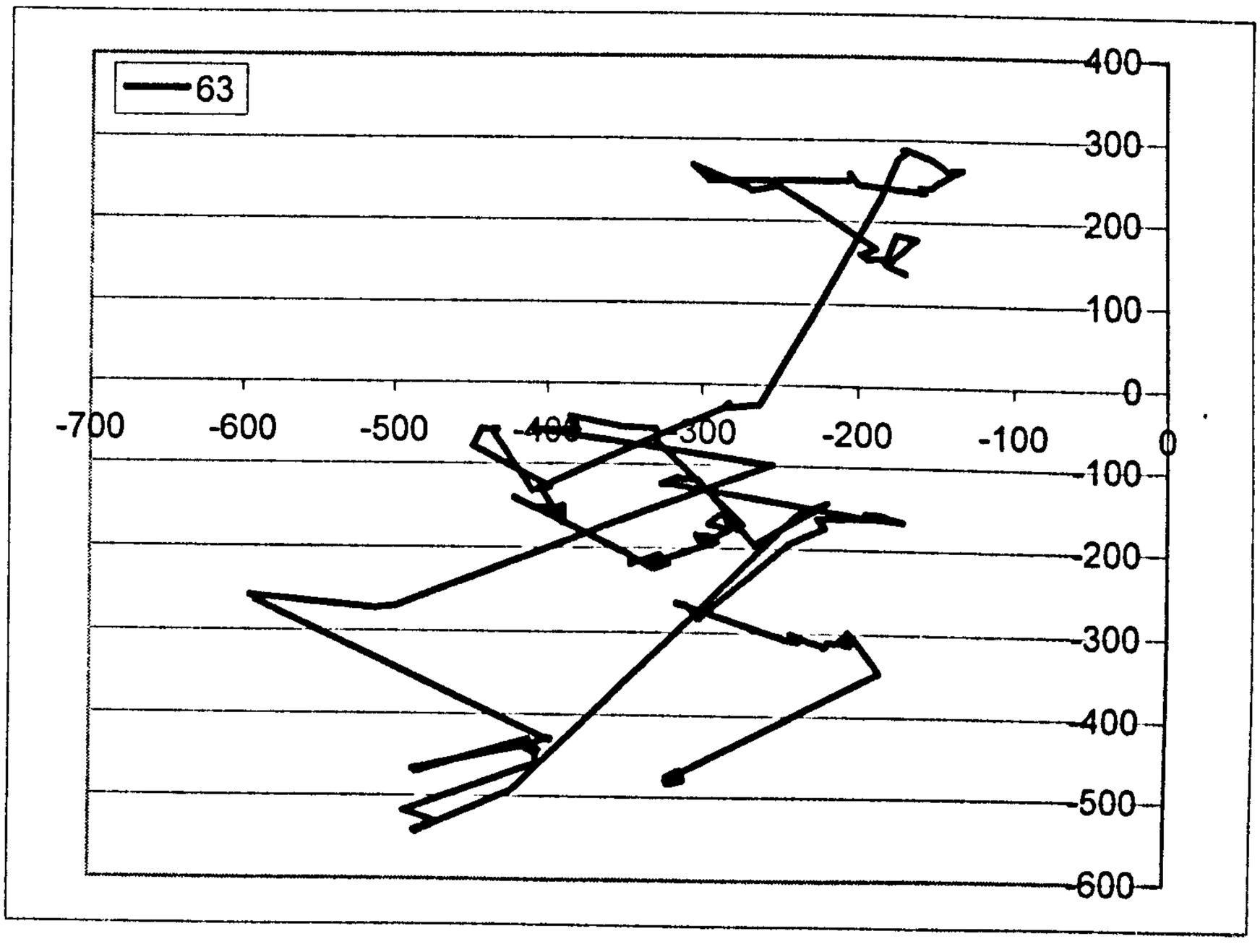
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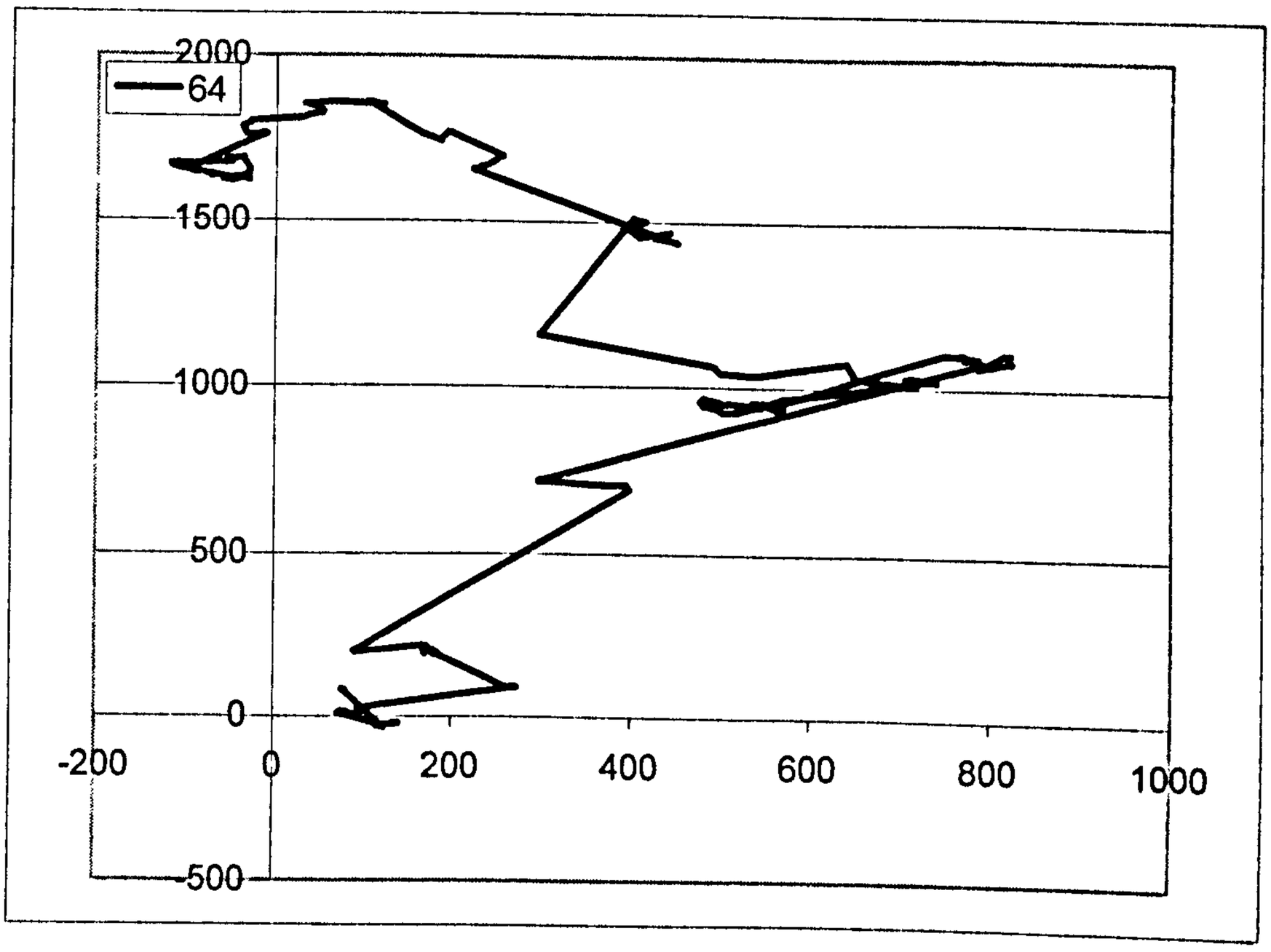
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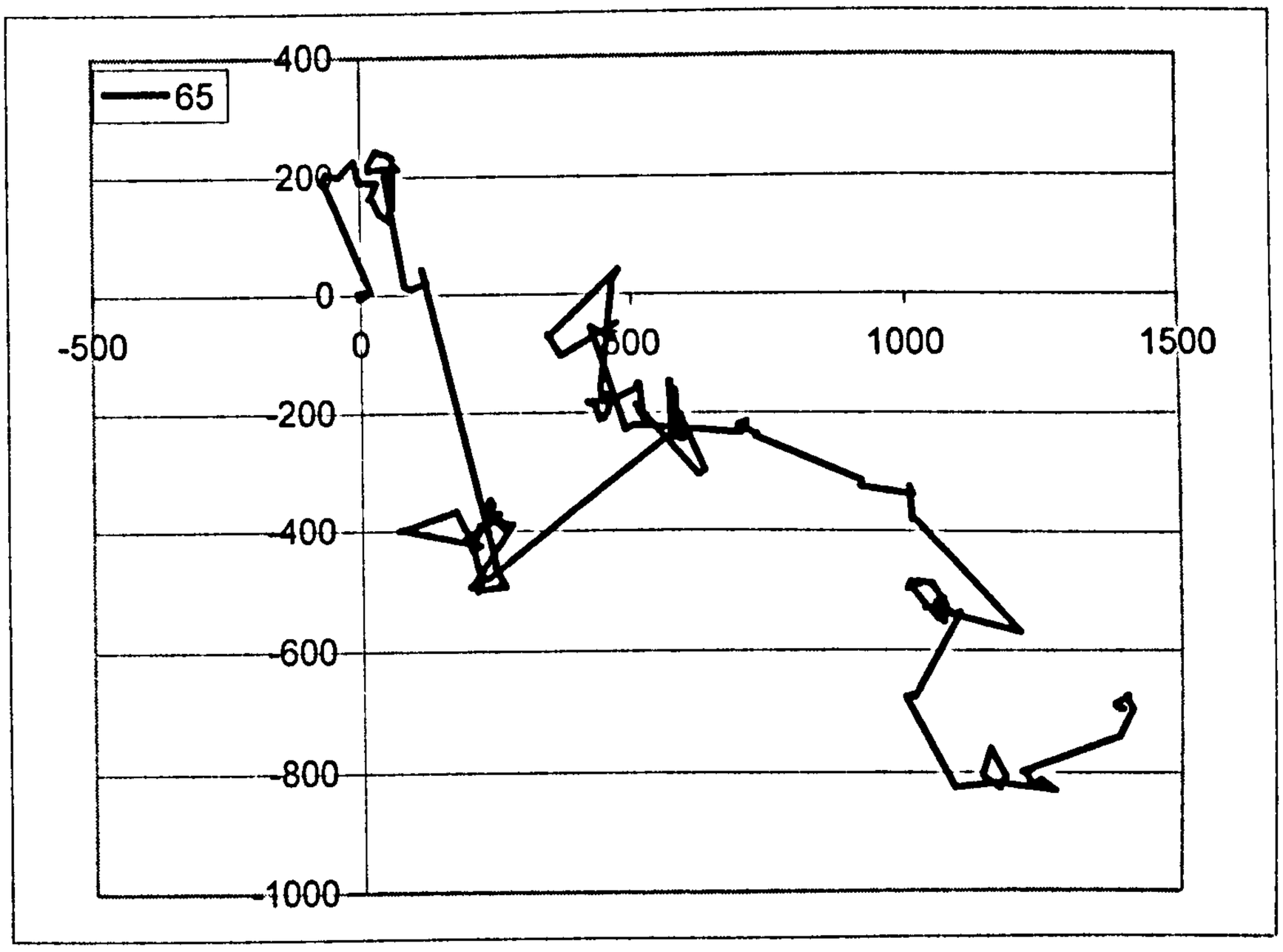
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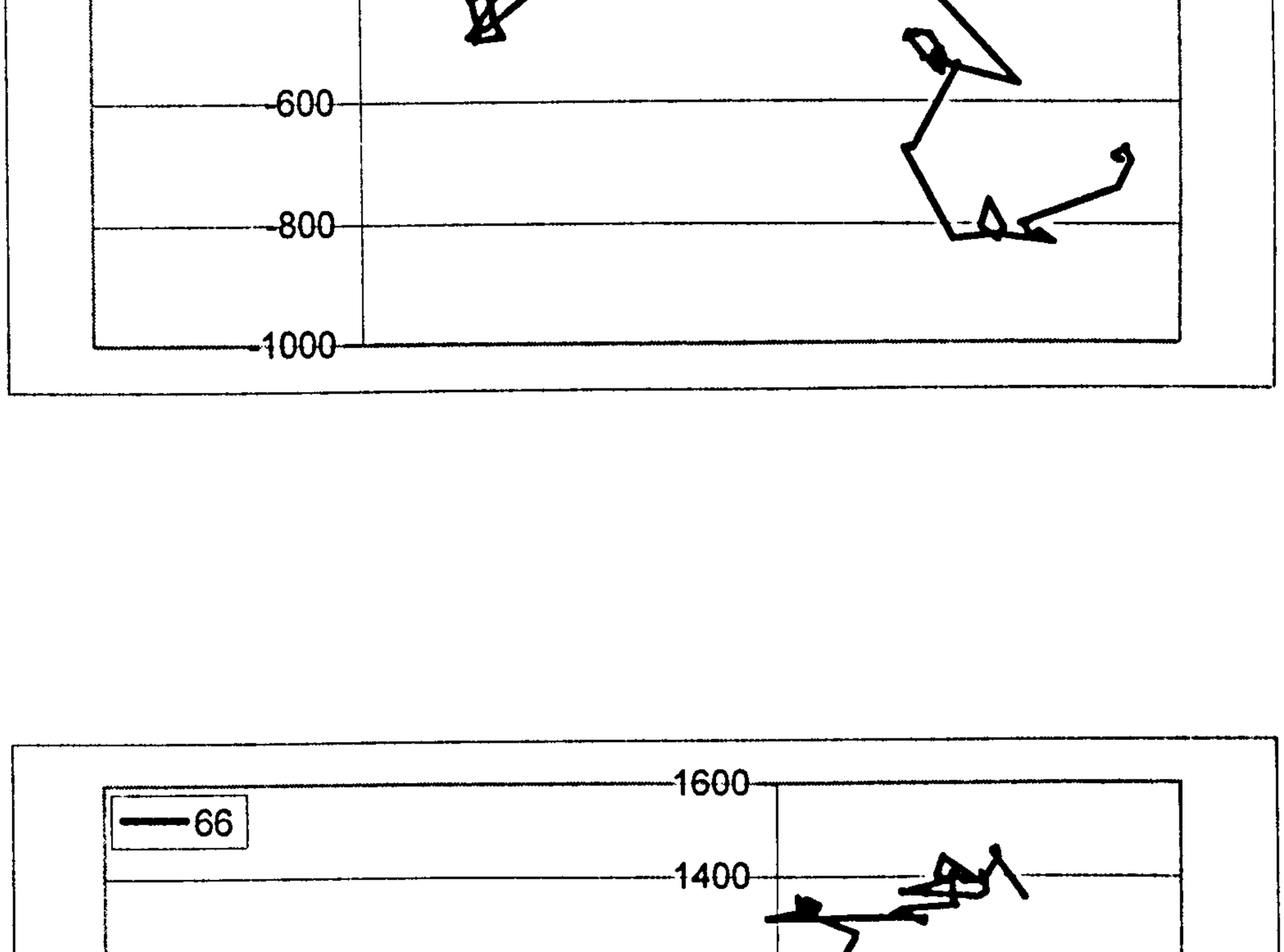
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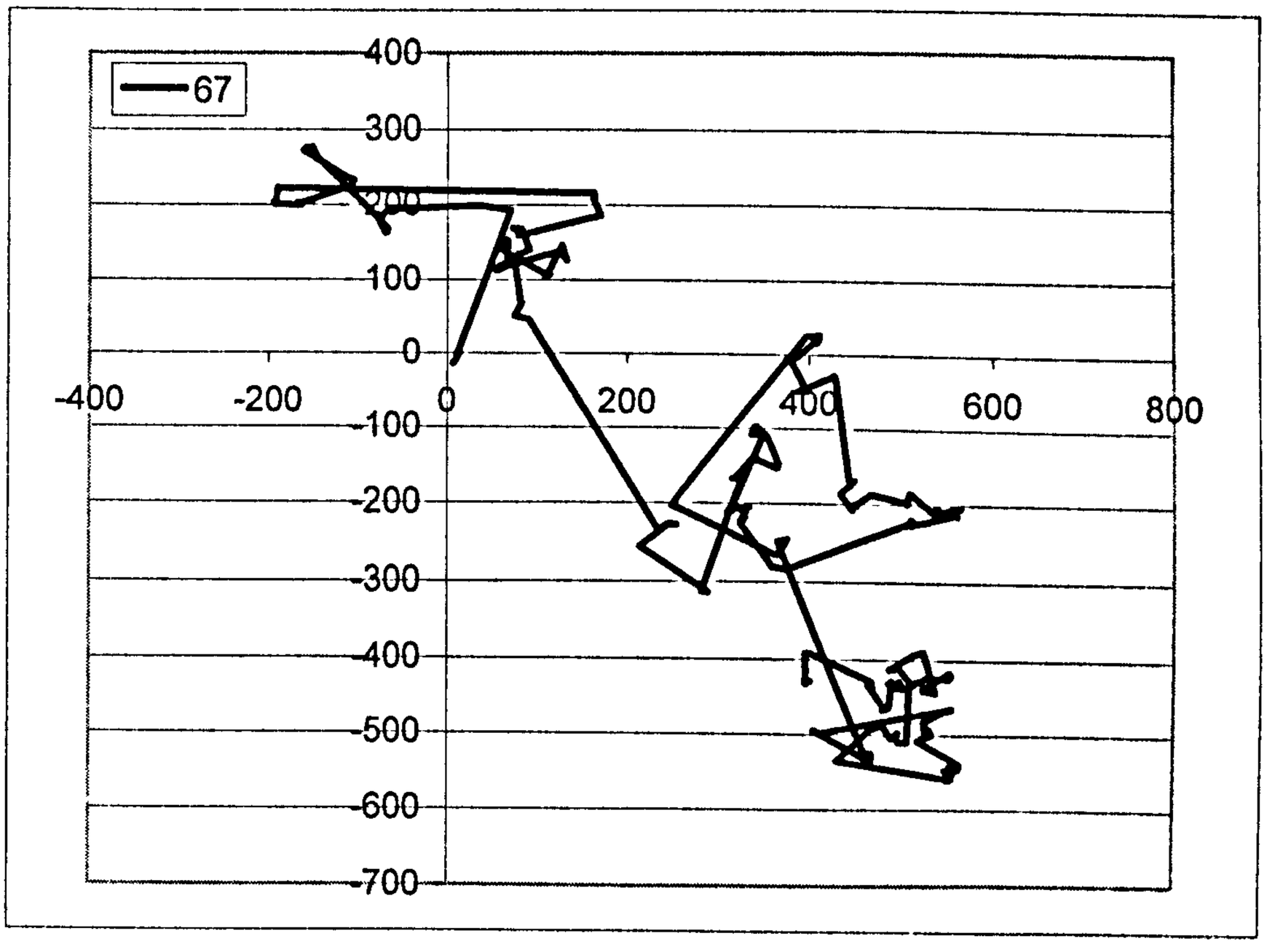
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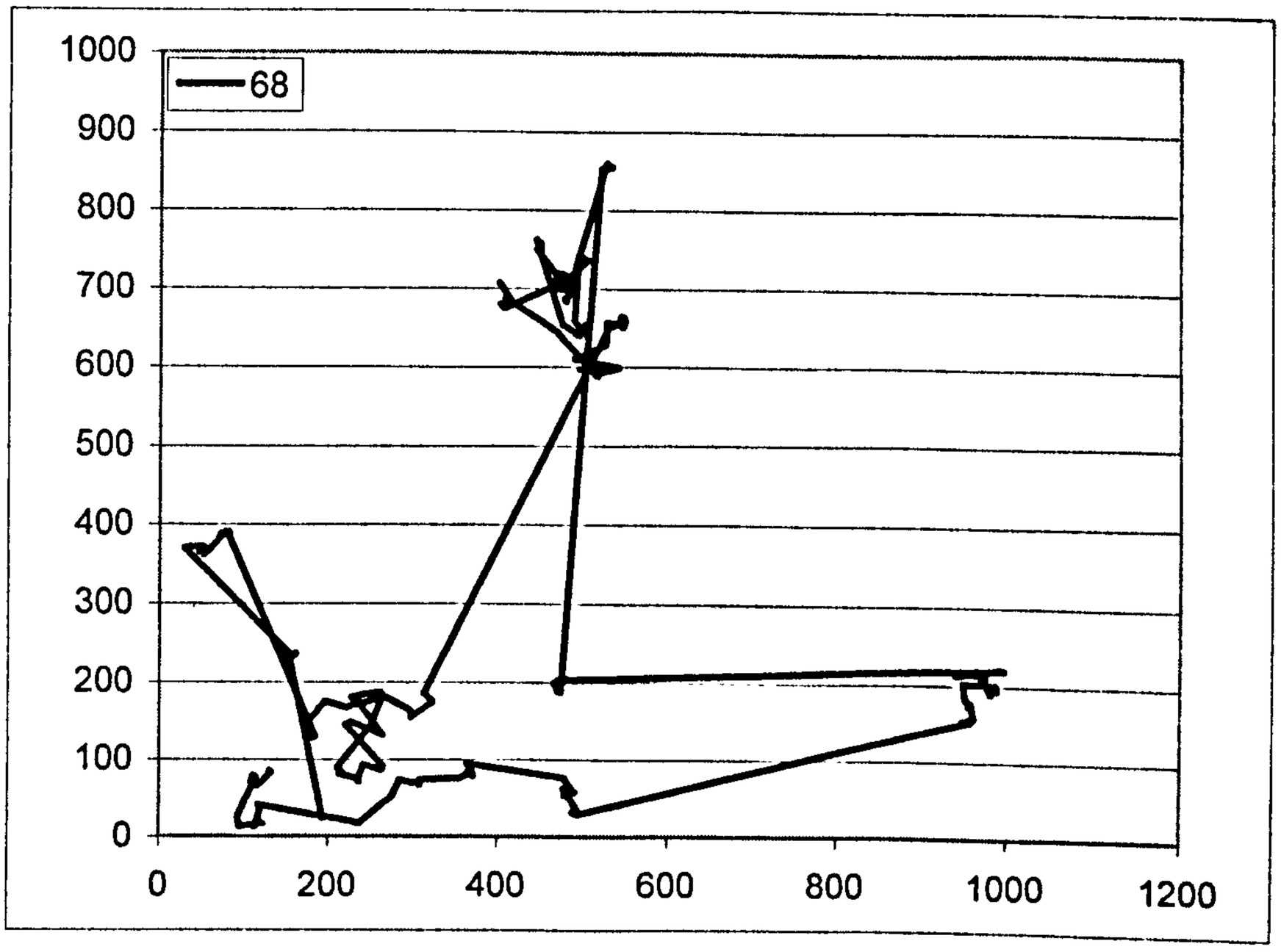
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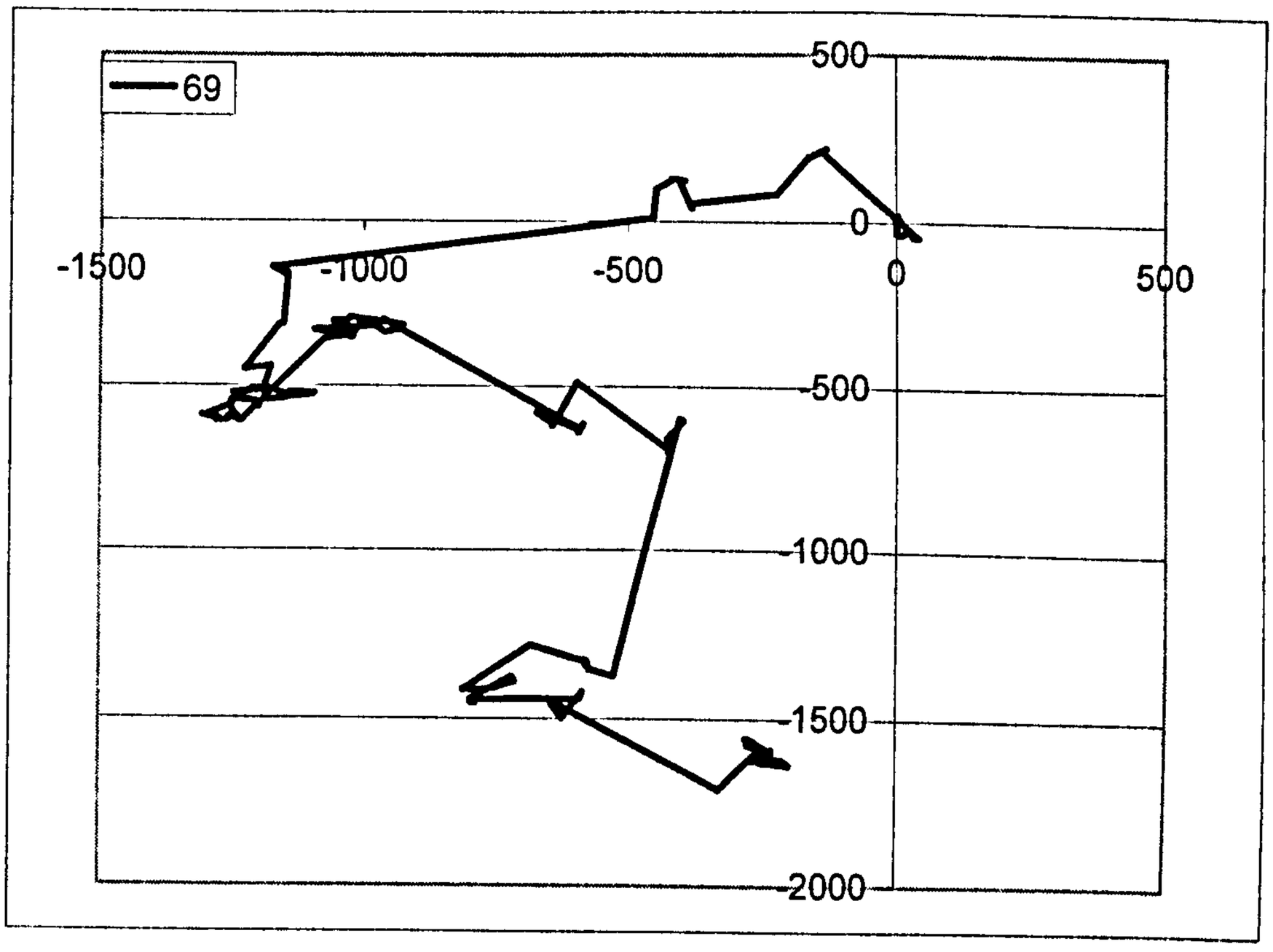
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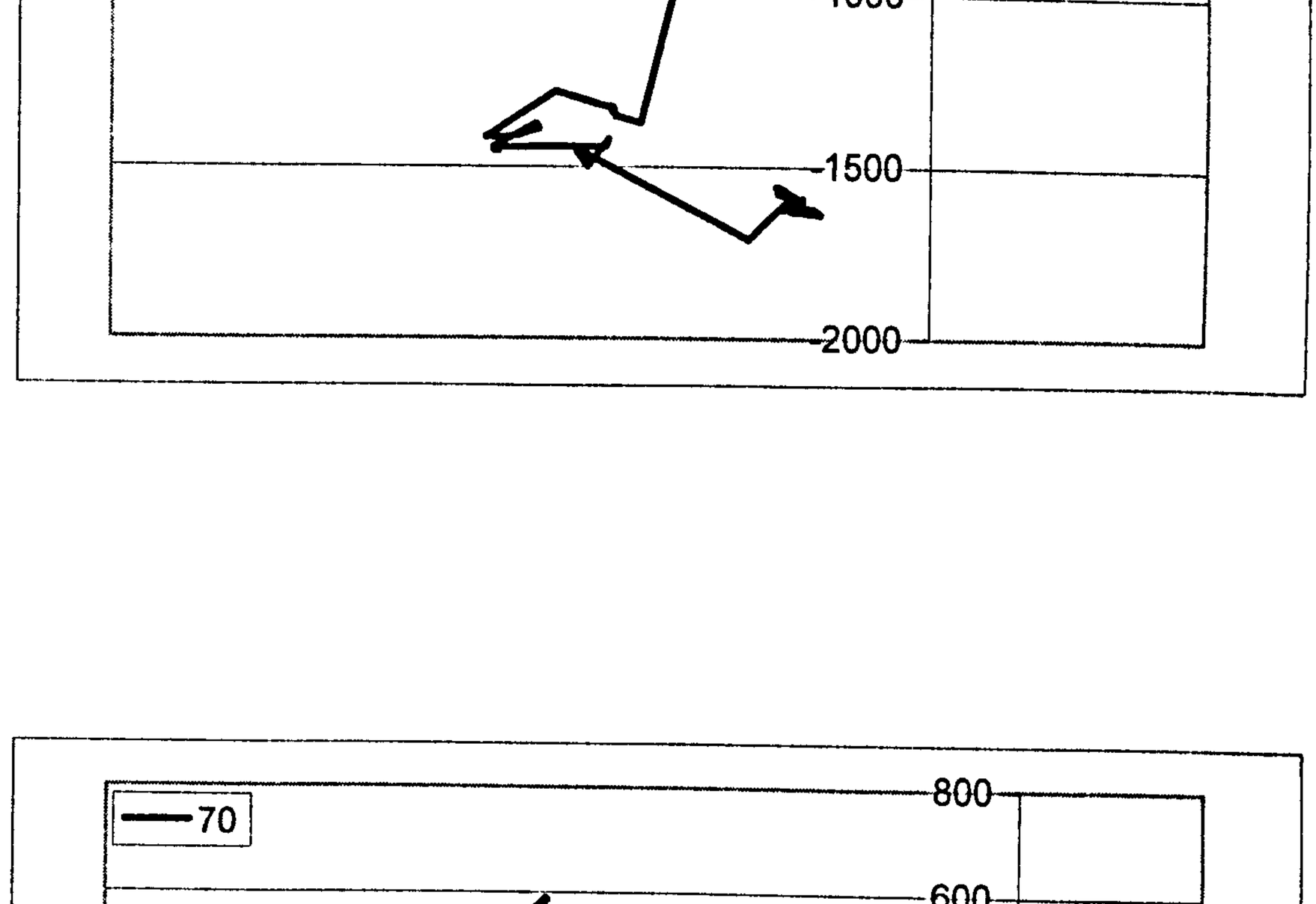
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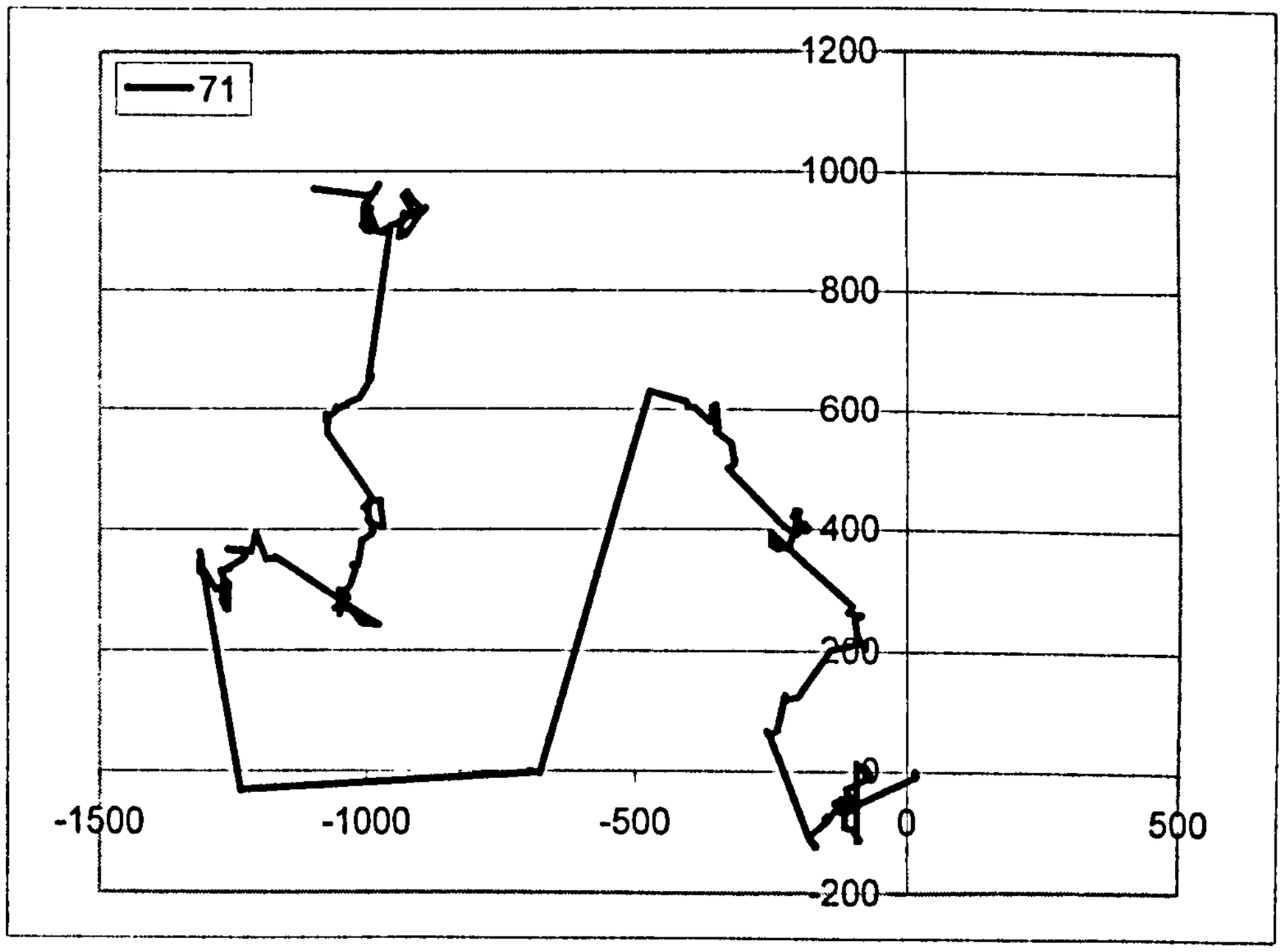
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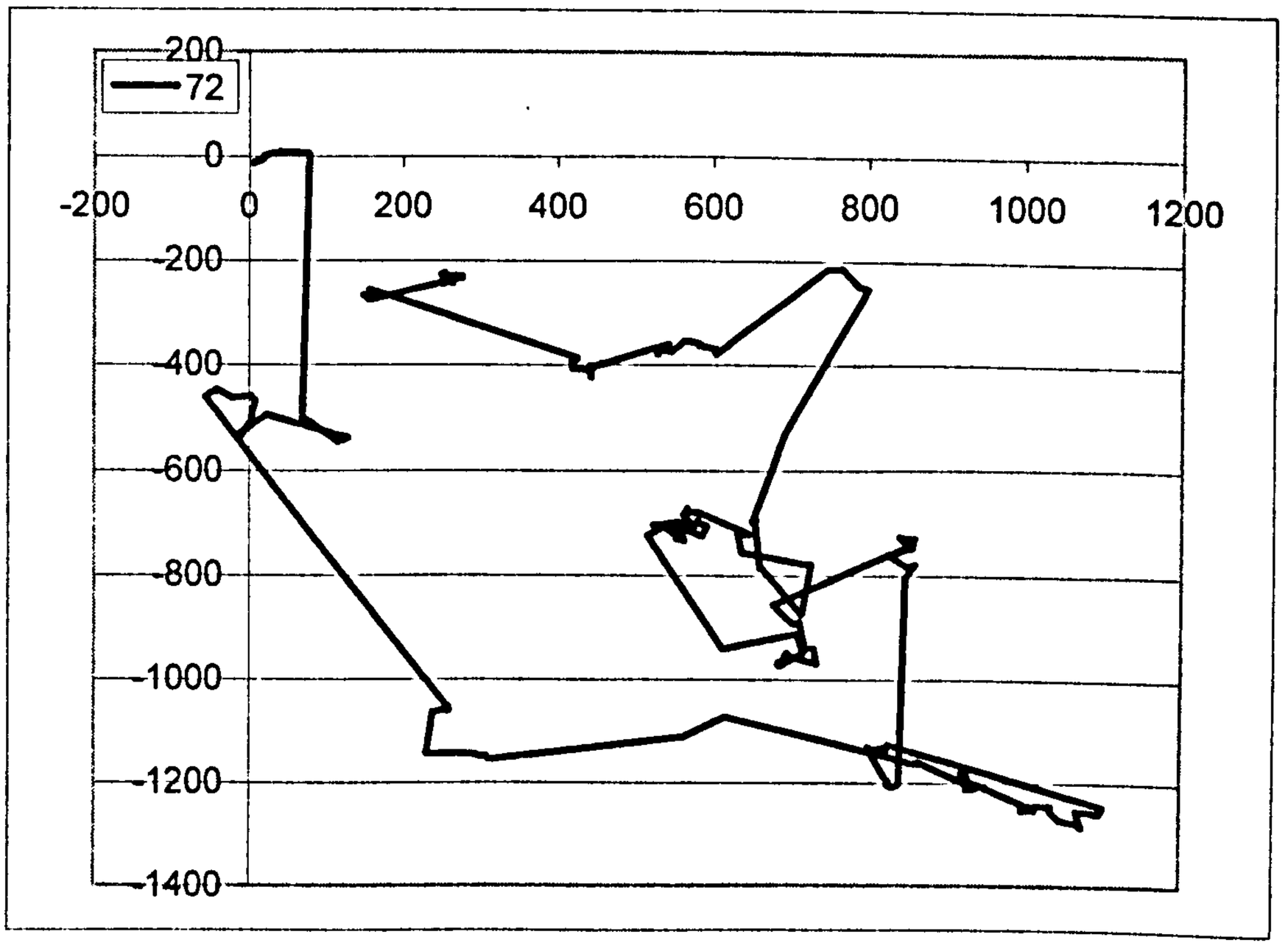
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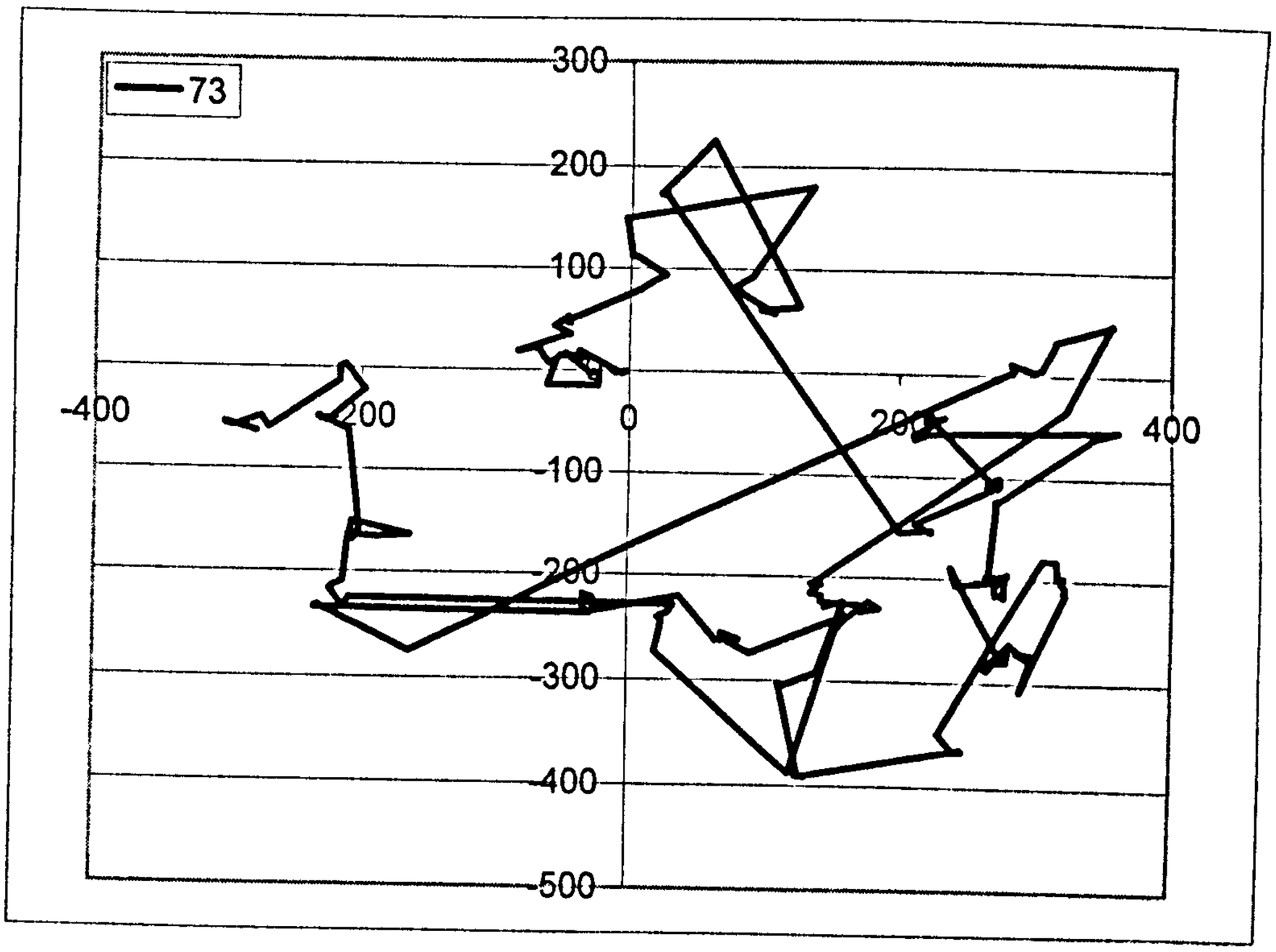
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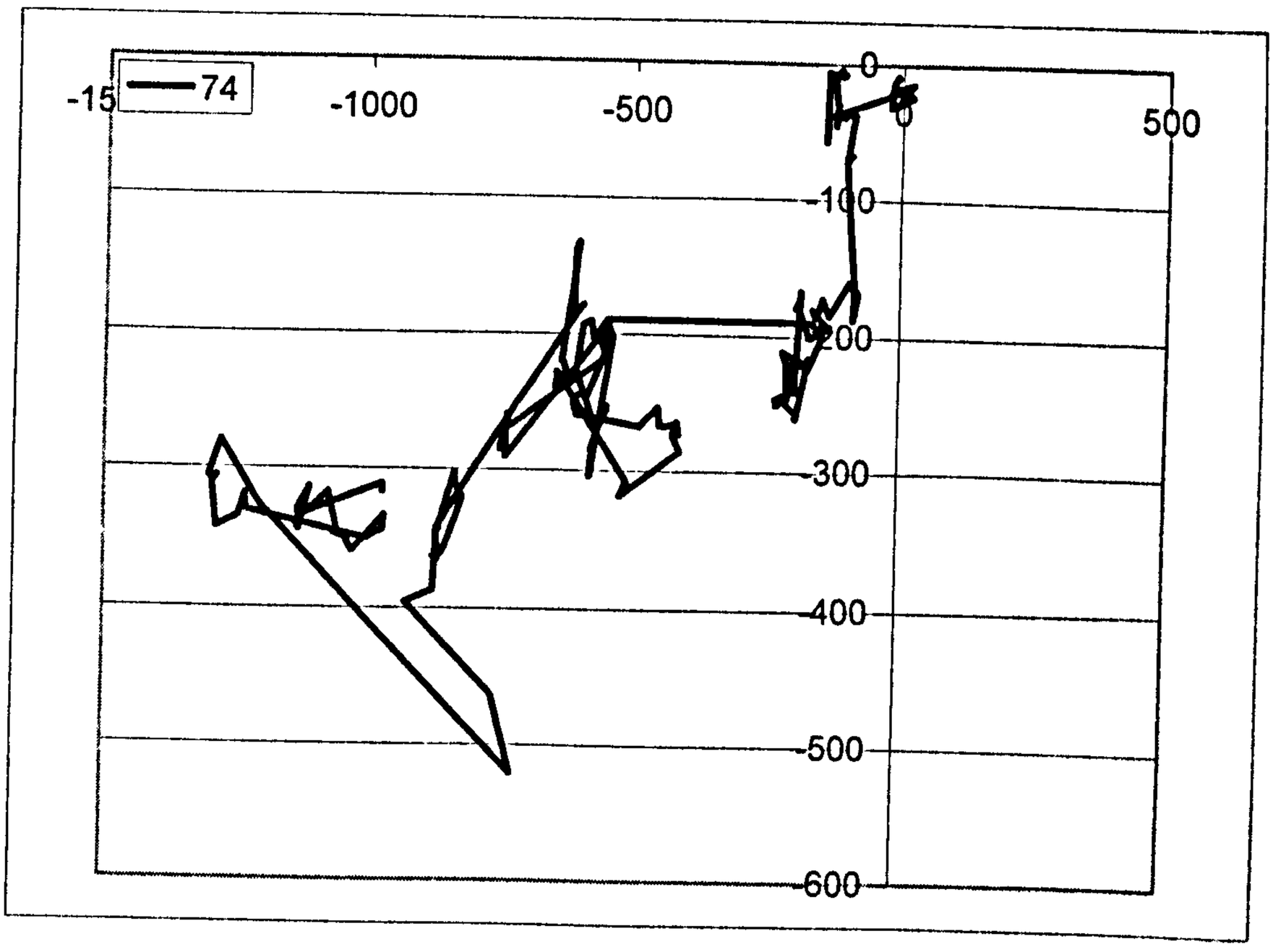
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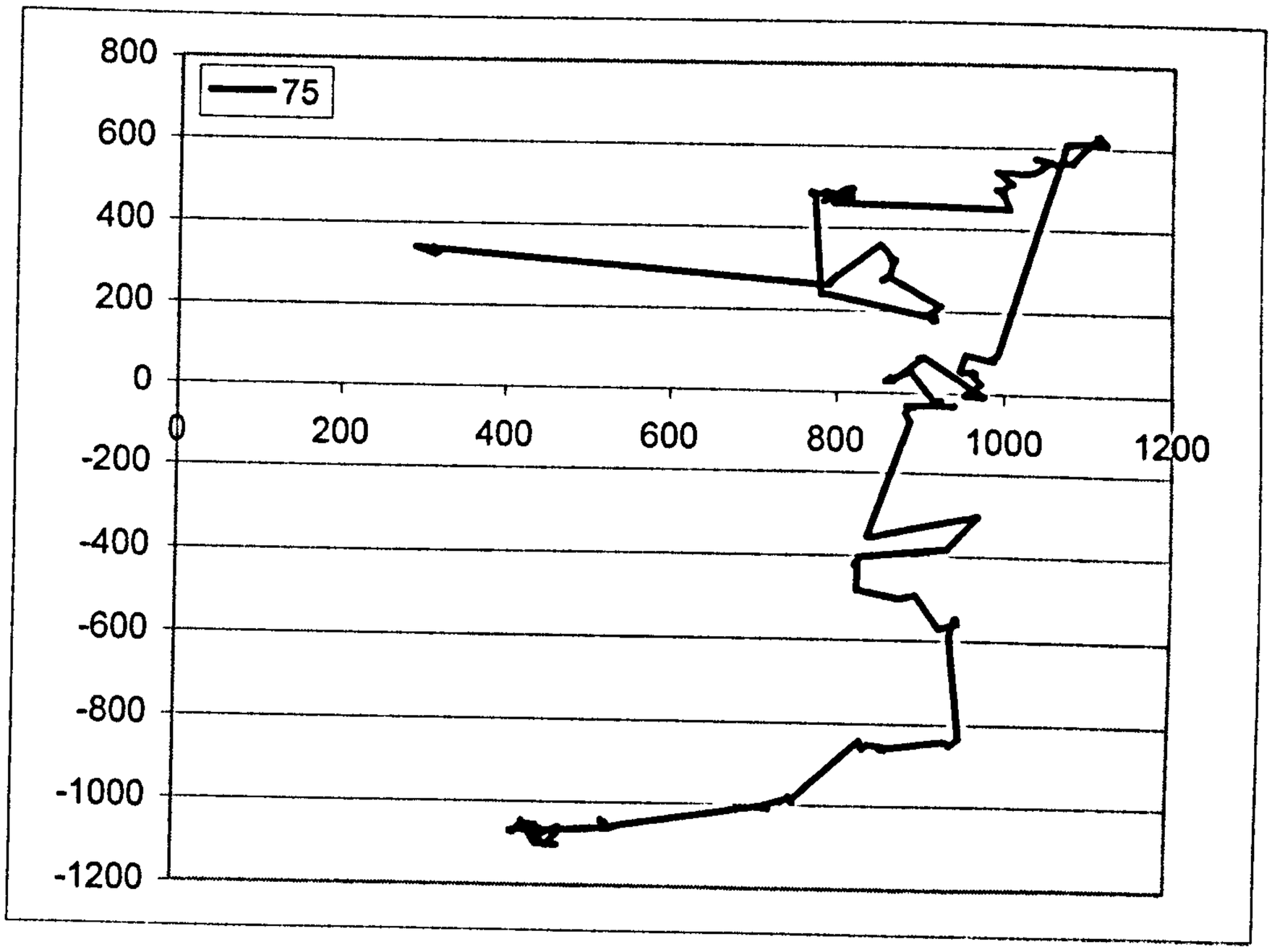
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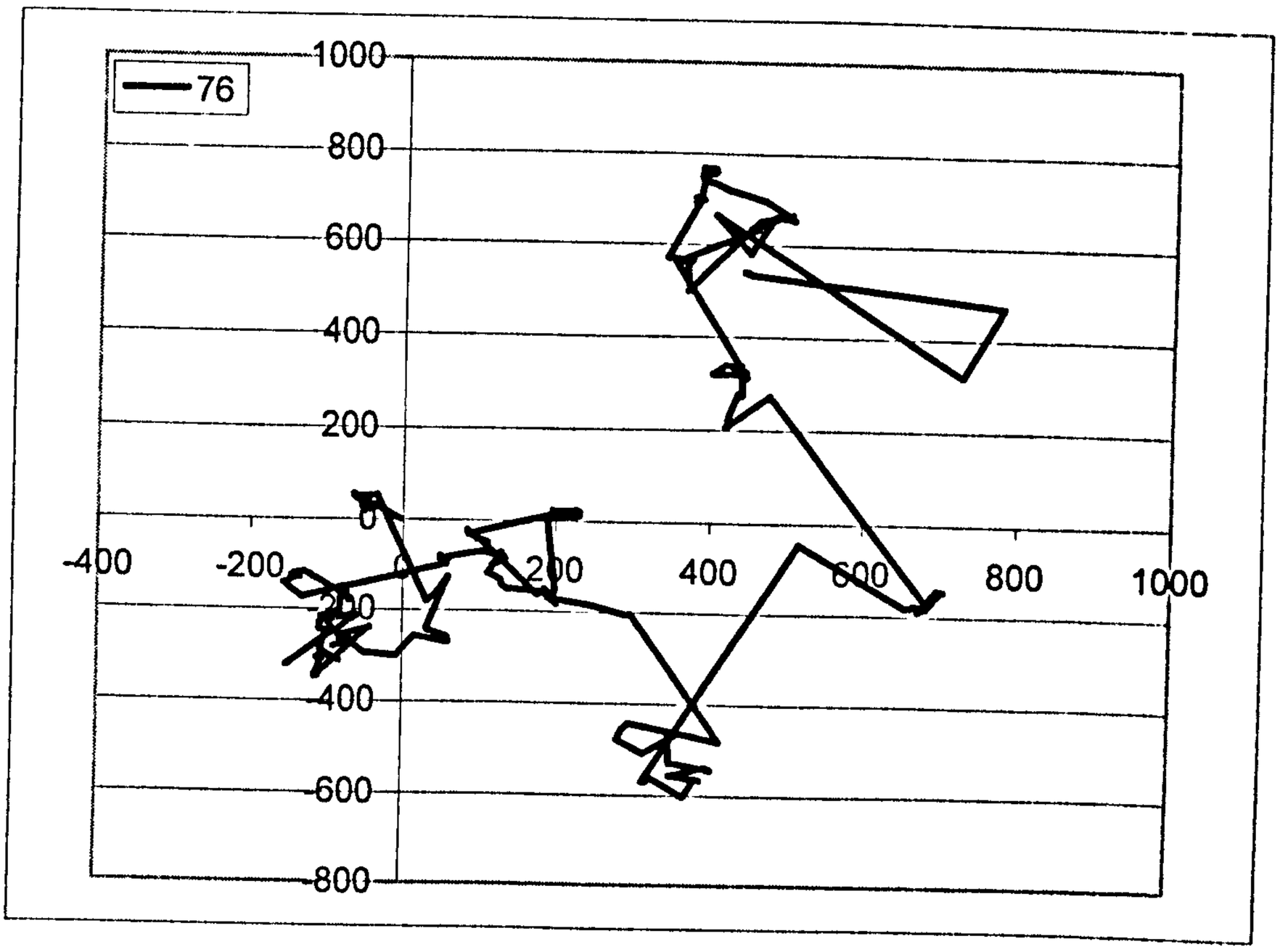
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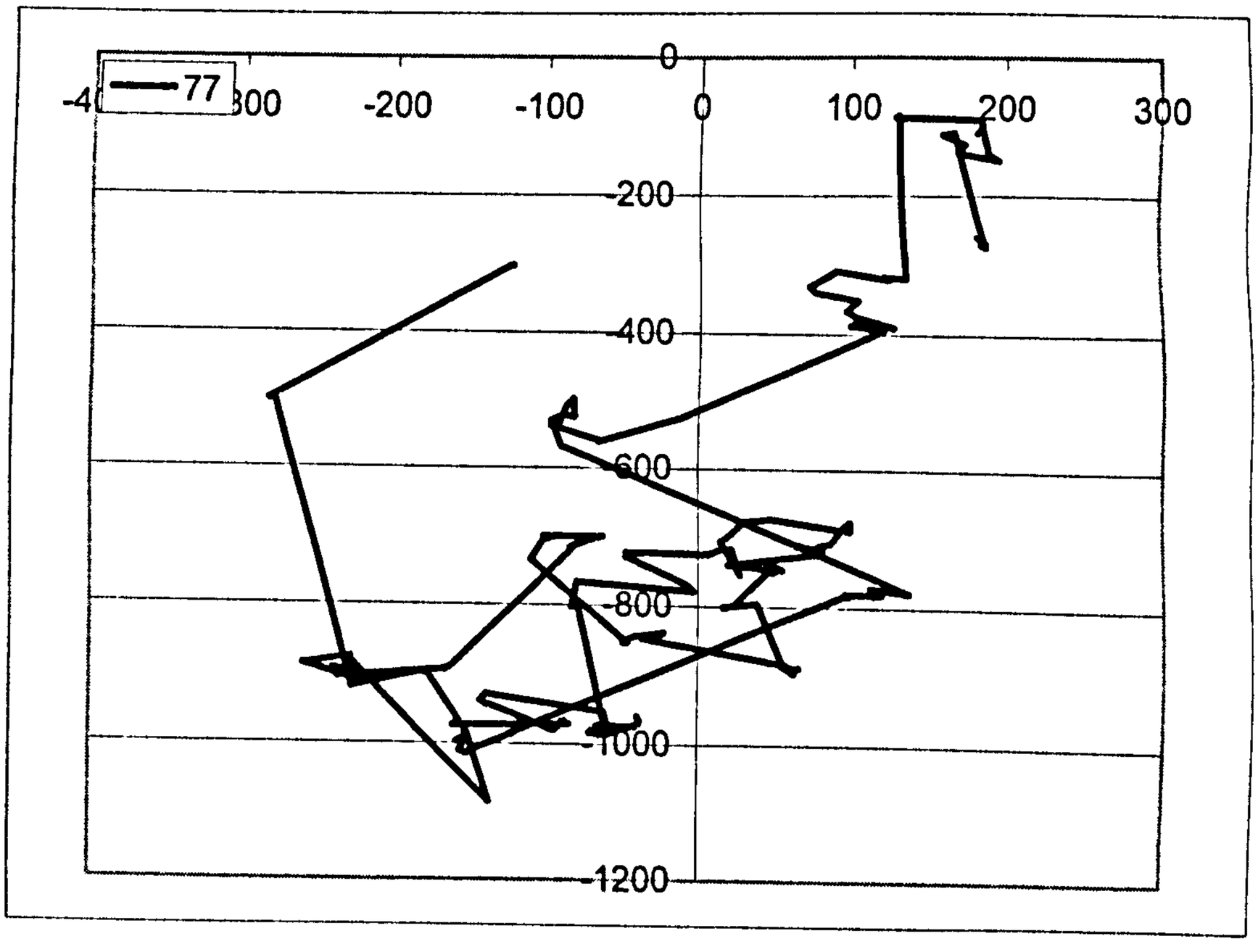
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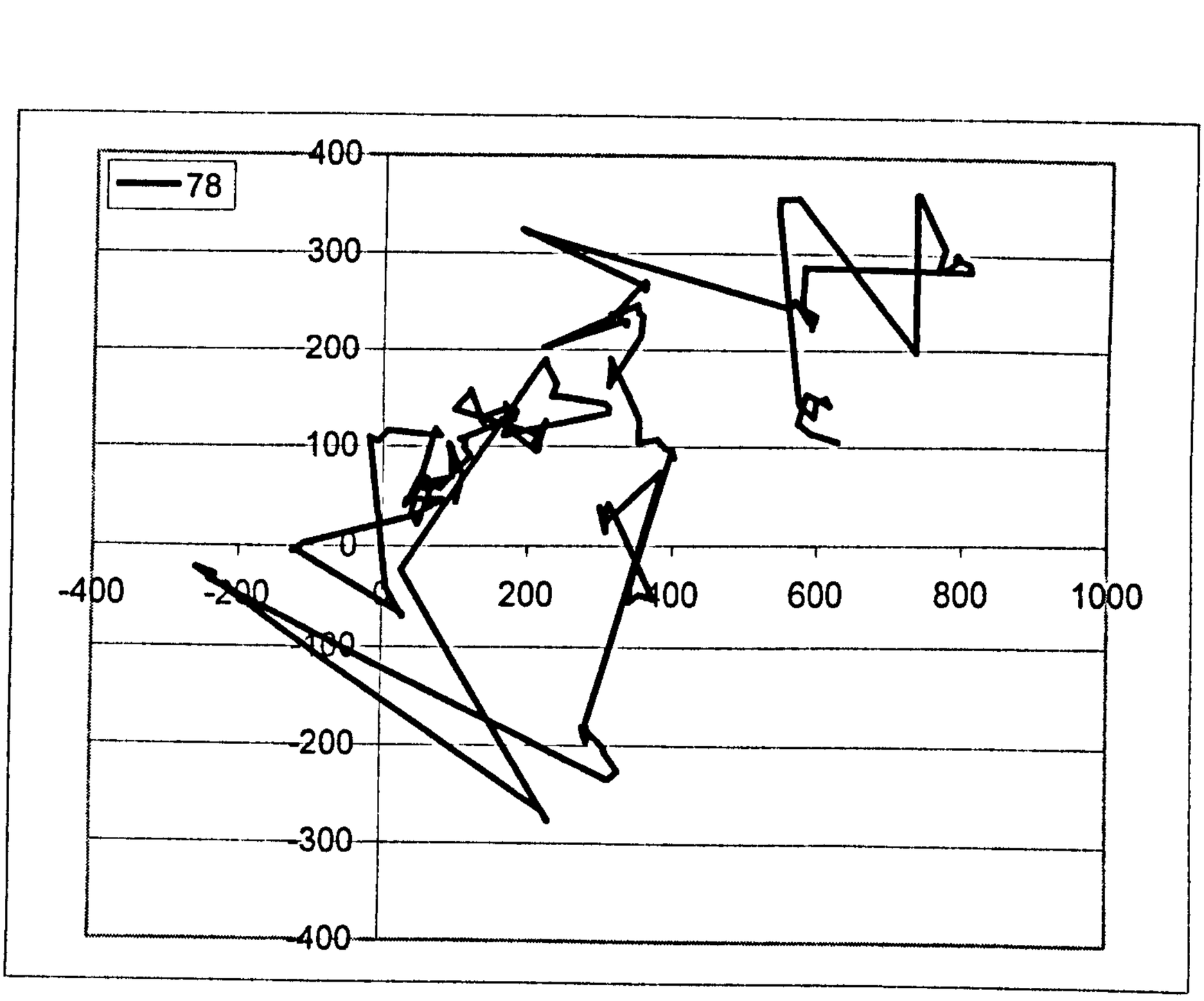
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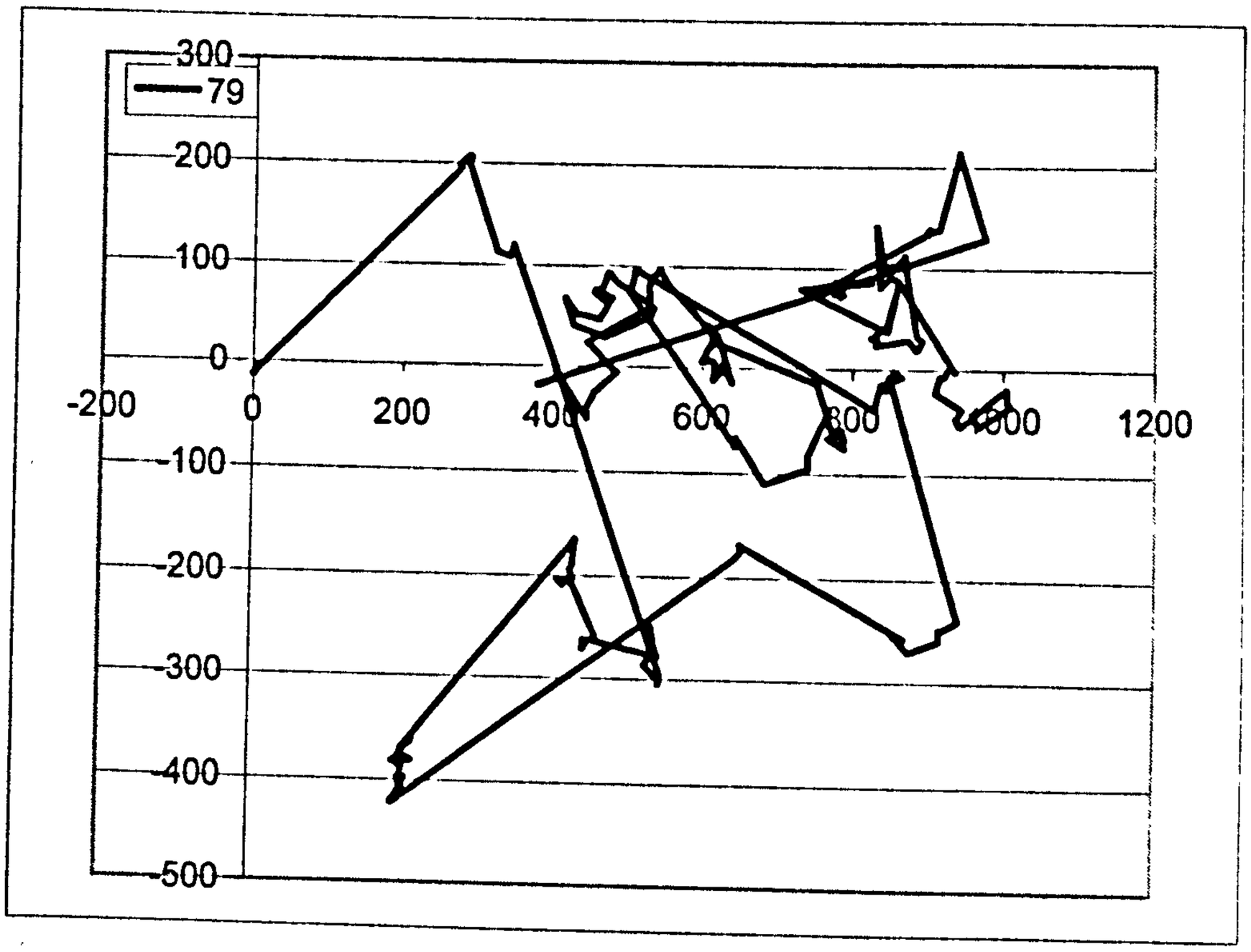
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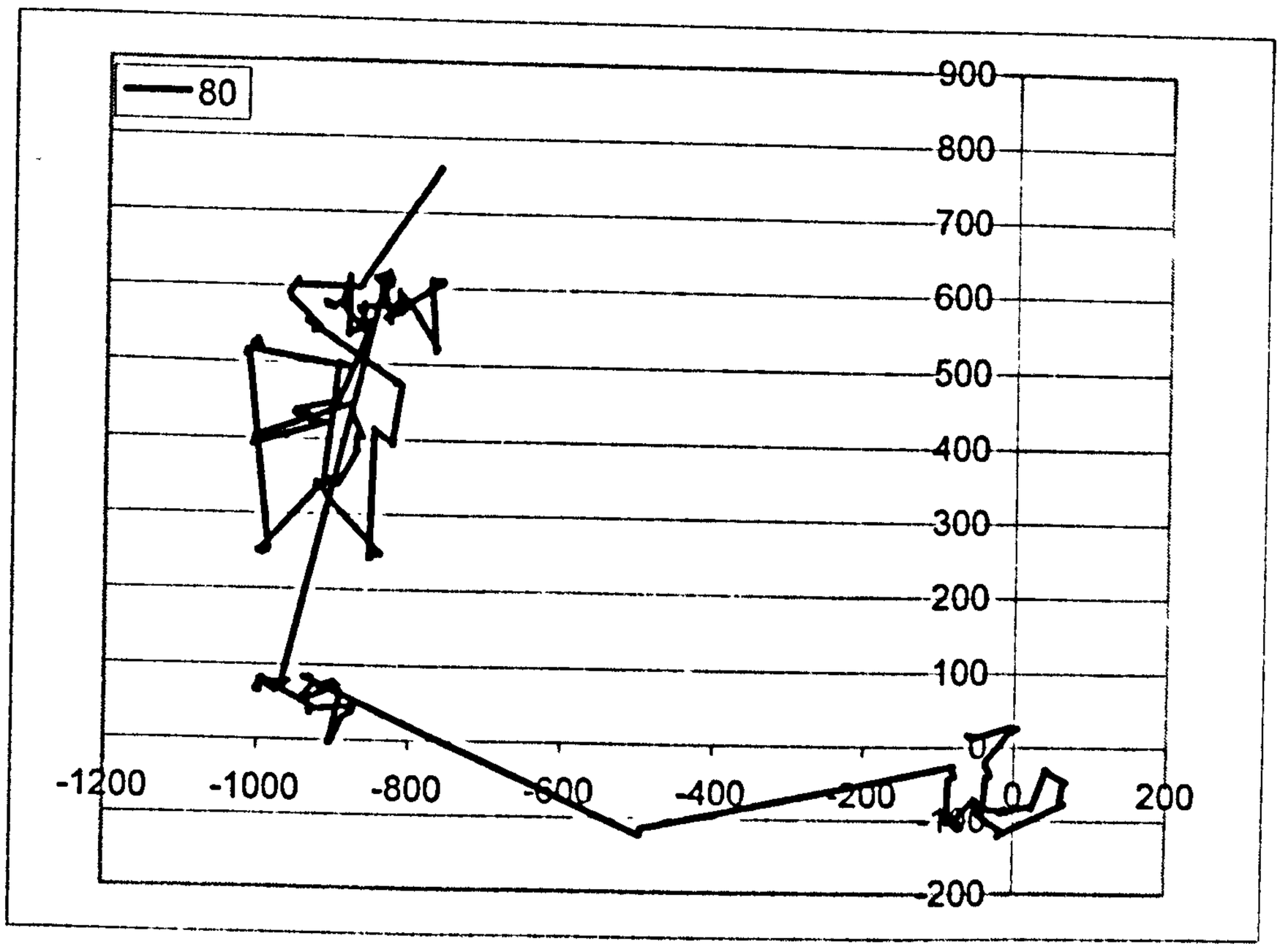
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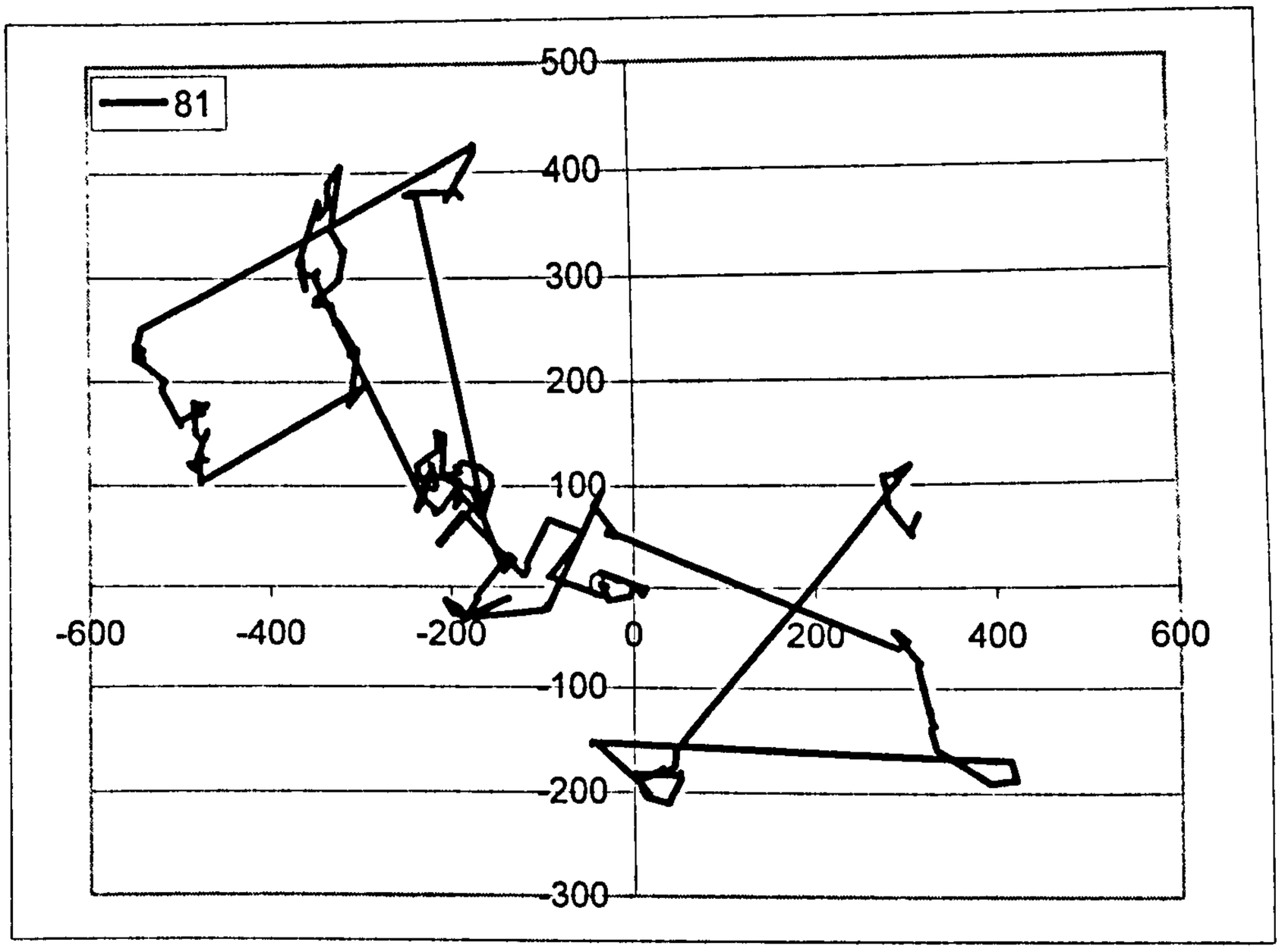
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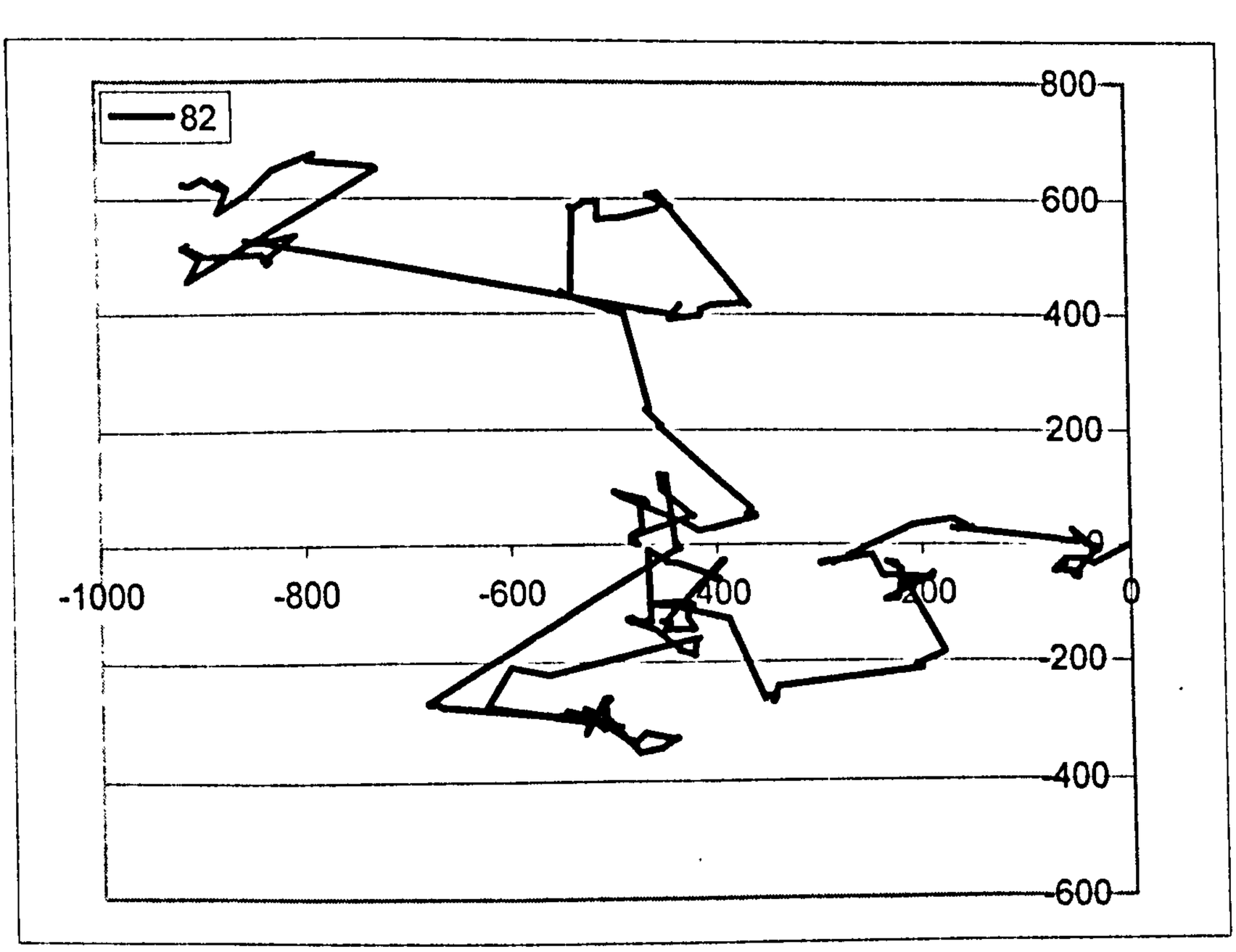
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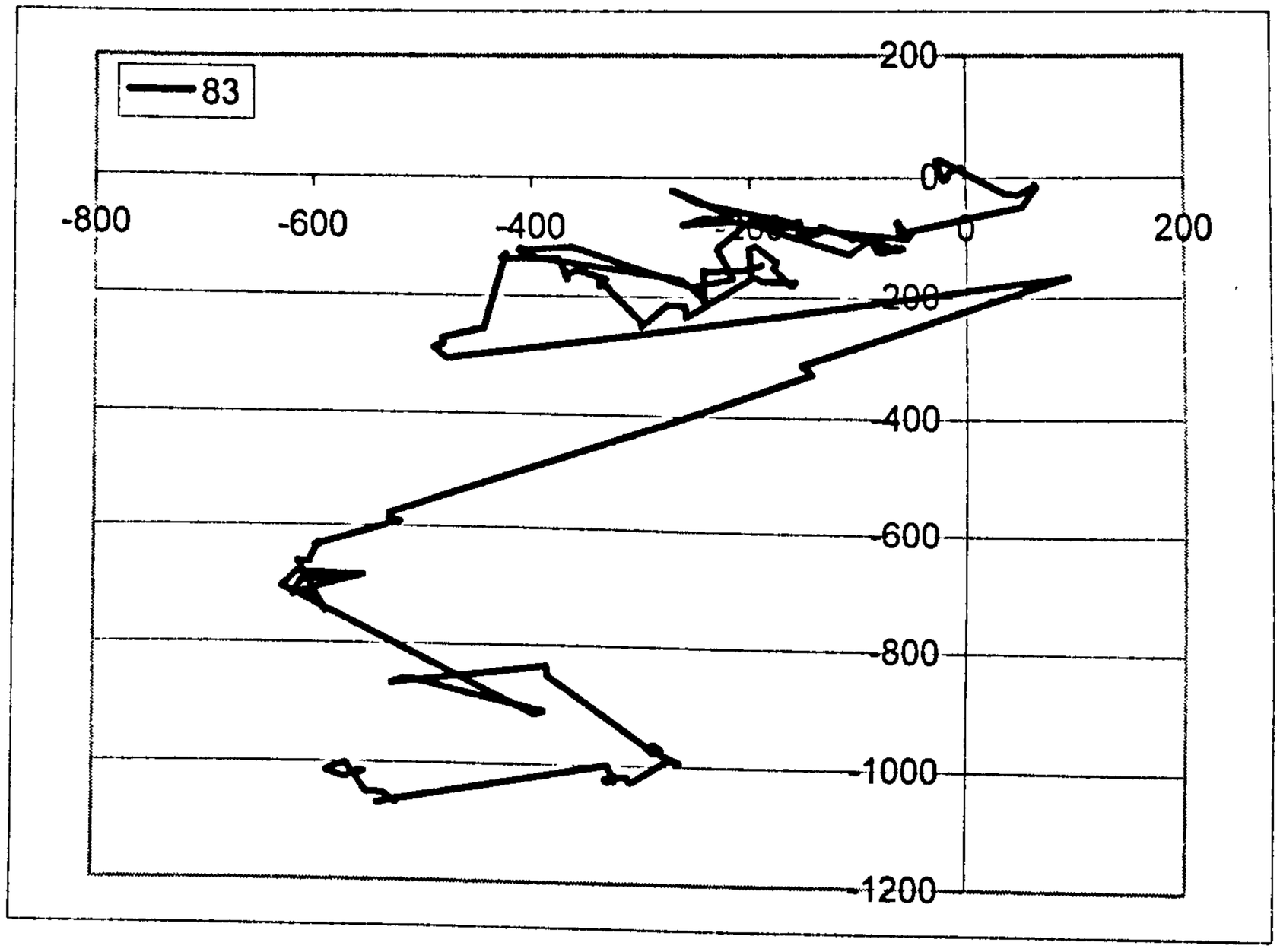
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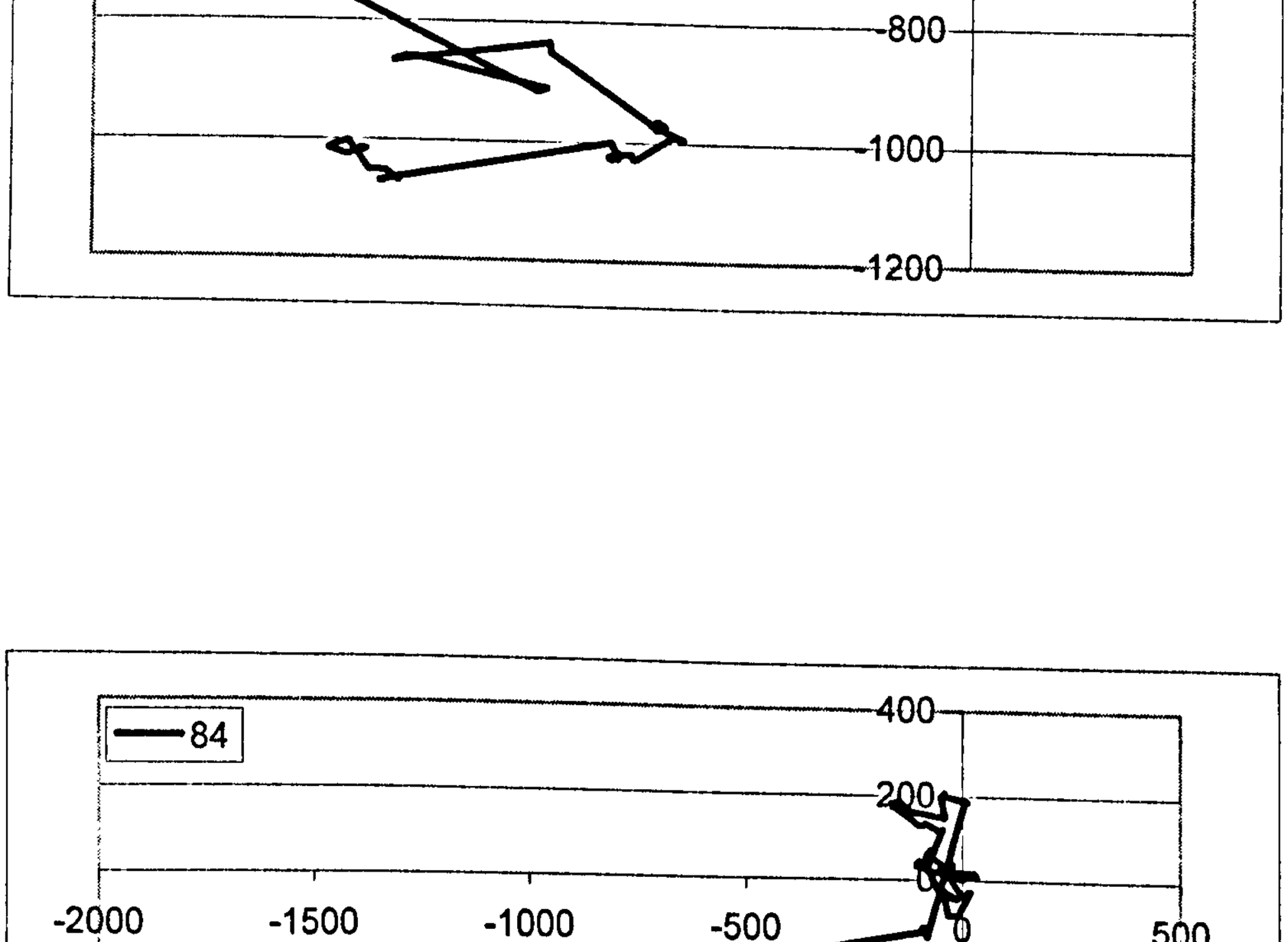
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